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# ULTRASTRUCTURE OF THE ASCUS APICAL APPARATUS IN HYMENOSCYPHUS AND OTHER GENERA OF THE HYMENOSCYPHOIDEAE (LEOTIALES, ASCOMYCOTINA)

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The ultrastructure of the ascus apical apparatus is compared in 19 species of the Hymenoscyphoideae, currently placed in seven genera. The ascus wall consists of an outer layer of two strata, and an inner layer of also two, and in one species of three strata. At the apex only the inner layer increases in thickness. On the basis of the general morphology and PA-TCH-SP reactivity pattern of the apex five main groups are recognized. A further division into subgroups is also outlined. The most important diagnostic features used in the analysis are the relative development and the reactivity pattern of the apical thickening, the occurrence of an annular protrusion, the structure and the reactivity pattern of the annulus, and the apex maturation pattern. In addition to the electron micrographs diagrammatic schemes are given to illustrate the author's interpretation.

The species studied are thus arranged as follows: Group 1a. *Hymenoscyphus caudatus*, *H. fructigenus*, *H. salicellus*, *H. salicinus*, and *Bisporella pallescens*; 1b. *H. imberbis* and *Phaeohelotium subcarneum*; 1c. *H. consobrinus*, *H. repandus*, and *Crocicreas pallidum*; 1d. *Discinella boudieri*; Group 2a. *H. herbarum*; 2b. *Pezizella gemmarum*; 2c. *Chlorociboria aeruginascens* and *Pezizella alniella*; 2d. *Crocicreas cyathoideum* [var. *cyathoideum*]; Group 3. *Bisporella sulfurina*; Group 4. *Cudoniella clavus* var. *grandis*; Group 5. *Cudoniella acicularis*.

Most fundamental are considered firstly the position of the annulus in the apical thickening, either partly (groups 1, 2, 4, 5) or fully (3) occupying the thickening, either associated (2, 4, 5) or not associated with an annular protrusion (1), and secondly the dehiscence mechanism, either an eversion of the annulus over an angle of about 90° (1, 2, 3?, 4) or a two-step mechanism (5) previously undescribed in Leotiales. The absence of an amyloid reaction in the apex, which is a diagnostic feature in *Cudoniella acicularis* and *C. clavus* var. *grandis*, is based on two fundamentally different structures in these species. The apex in the last mentioned fungus closely resembles that in *Ombrophila violacea*, while the apex in *C. acicularis* is unique in general morphology and dehiscence mechanism.

The ultrastructural data of the apical apparatus are found to correlate with characters of excipulum anatomy, especially in the genera *Hymenoscyphus* and *Bisporella*. Their importance in segregating more natural genera from large ill-defined genera like *Hymenoscyphus* or *Pezizella* is discussed.

## INTRODUCTION

The structure of the ascus is of paramount importance in the classification of the higher taxa in the Ascomycotina (Boudier, 1879, 1907; Nannfeldt, 1932; Korf, 1973; Eriksson & Hawksworth, 1987, 1988). In contrast, its influence at lower taxonomic levels is lim-

ited. Several students of ascomycetes have emphasized the possible significance of the structure of the ascus for the delimitation of families and genera (Luttrell, 1951; Chadefaud, 1973; Beckett, 1981). The light optical studies on ascus structure in the Leotiales S. Carp. were valuable as far as they were focussed on structures with dimensions well above the limit of the resolving power of the light microscope. For the study of structures in the ascus with dimensions close to or under this limit, such as the basal septum and the apical apparatus, only the transmission electron microscope offers the appropriate resolving power. As yet this instrument has been used by few taxonomists to study a limited number of species of the Leotiales (Schoknecht, 1975; Bellemère, 1975, 1977; Benny et al., 1978; Bellemère et al., 1987; Verkley, 1992, 1993).

Monographers of Leotiales are still faced with a scarcity of distinctive characters useful to define generic concepts. Especially for the circumscription of genera in the family Leotiaceae Corda they have to depend largely on characters most of which also occur outside the group of species to be considered. Some genera are ill-defined and an extremely large number of species has been referred to them over the years. *Hymenoscyphus* S.F. Gray and *Pezizella* Fuckel thus became classical examples of a 'waste-basket genus' (Korf, 1973).

The genus *Hymenoscyphus* as interpreted by most authors for many years (Dennis, 1964, 1978; Dumont, 1981) and most recently by Lizoñ (1992), shows considerable variation in the anatomy of the ectal excipulum and in ascospore characters, e.g. shape and presence of 'cilia'. Efforts to separate from the large genus *Hymenoscyphus* a number of new genera were not successful (Dennis, 1956, 1964; Dumont, 1981). Several species of *Bisporella* Sacc., *Pezizella*, and *Phaeohelotium* Kanouse have been considered more closely related to, or congeneric with, certain species of *Hymenoscyphus* (Baral & Krieglsteiner, 1985). Baral & Krieglsteiner (1985) have proposed major systematic changes under the influence of their light microscopic observations of the ascus apex. For reasons mentioned above this is a less fortunate approach. It illustrates the great need for additional characters that are less variable at the genus level and of a comparatively conservative evolutionary nature. Ultrastructural studies of the apical apparatus may provide a complex of such characters. The present investigation of eight species of *Hymenoscyphus* is an effort to determine the variation of this character complex, firstly within a group of species considered structurally more similar to the type species, *H. fructigenus* (Bull.: Fr.) S.F. Gray, than others, and secondly between this group and structurally different species currently referred to this genus.

The variation in ultrastructure of the ascus wall and the apical apparatus in other genera of the Hymenoscyphoideae sensu Korf (1973) is also practically unknown. Therefore, the ultrastructure of the ascus apical apparatus of 11 selected species from the genera *Bisporella*, *Cudoniella* Sacc., *Chlorociboria* Seaver ex Ramamurthi et al. emend. Dixon, *Discinella* Boud., *Crocicreas* Fr., and *Pezizella* were studied. The data are compared with those obtained in earlier studies on Sclerotiniaceae and Ombrophiloideae sensu Dennis (Verkley, 1992, 1993). They are discussed in relation to other characters like those pertaining to the structure of the excipulum and the ascospores.

## MATERIALS AND METHODS

Fresh material was collected in the field. Parts of fruit-bodies were fixed for 3 hours using 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C. After washing in buffer the material was postfixed for 1 hour using 1% osmium tetroxide in cacodylate buffer at room temperature. During dehydration the material was stained with 1% uranyl acetate dissolved in 30, 50, 70, and 96% ethanol in water (5 minutes for each grade). Then the material was embedded in Epon 3/7. Ultrathin sections were cut using a diamond knife on a Reichert Jung Ultracut E ultratome.

Sections were picked up on 200 mesh gold grids and treated for periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) as described by Verkley (1992) and modified from Thiéry (1967).

Preparations were examined using a Philips EM 300 or Jeol JM 1010 electron microscope at 60 kV.

In the following list details are given about the origin of the collections, deposited in Leiden (L).

*Bisporella pallescens* (Pers.) S. Carp. & Korf. 'Oud Poelgeest', Oegstgeest, prov. Zuid-Holland, the Netherlands, on shredded wood, May 1992, *G. Verkley 126*.

*Bisporella sulfurina* (Quél.) S. Carp. Windesheim, Zwolle, prov. Overijssel, the Netherlands, on wood, Oct. 1990, *Piepenbroek 1834*; Oostvaardersplassen, Lelystad, prov. Flevoland, the Netherlands, on dead stems of *Epilobium*, July 1991, *G. Verkley 87*.

*Chlorociboria aeruginascens* (Nyl.) Kanouse ex Ramamurthi et al., Fôret de Citeaux, Bagnot, dép. Côte d'Or, France, on dead wood, Oct. 1990, *J. van Brummelen 7957*.

*Crocicreas cyathoideum* (Bull.: Fr.) S. Carp. [var. *cyathoideum*]. Windesheim, Zwolle, prov. Overijssel, the Netherlands, on dead stems of Umbelliferae, May 1990, *Piepenbroek 1760*; Oranjepolder, Voorschoten, prov. Zuid-Holland, the Netherlands, on dead stems of *Urtica*, Apr. 1992, *G. Verkley 123*.

*Crocicreas pallidum* (Velen.) S. Carp. Oranjepolder, Voorschoten, prov. Zuid-Holland, the Netherlands, on fallen petioles of *Fraxinus* and *Acer*, Oct. 1991, *G. Verkley 96*.

*Cudoniella acicularis* (Bull.: Fr.) J. Schroet. Finnån river, east of Femsjö, Smölland, Sweden, on soil and plant debris, Aug. 1989, *J. van Brummelen 7885*.

*Cudoniella clavus* (Alb. & Schw.: Fr.) Dennis var. *grandis* (Boud.) Dennis, Roode Beek, Vlodrop, prov. Limburg, the Netherlands, on dead wood, May 1990, *H. Huijser s.n.*

*Discinella boudieri* (Quél.) Boud. Fôret de St. Prix, Arnay-le-Duc, dép. Côte d'Or, France, on soil, Oct. 1990, *H. Marxmüller (J. van Brummelen 7975)*.

*Hymenoscyphus caudatus* (P. Karst.) Dennis. Oranjepolder, Voorschoten, prov. Zuid-Holland, the Netherlands, on dead herbaceous stems and fallen petioles of *Fraxinus*, July 1991, *G. Verkley 95*.

*Hymenoscyphus consobrinus* (Boud.) Hengstmengel. Hengforder Waarden, Olst, prov. Overijssel, the Netherlands, on dead herbaceous stems, June 1990, *Piepenbroek 1770*; Windesheim, Zwolle, prov. Overijssel, the Netherlands, on dead herbaceous stems, July 1990, *Piepenbroek 1790*.

*Hymenoscyphus fructigenus* (Bull.: Fr.) S.F. Gray. 'Oud Poelgeest', Oegstgeest, prov. Zuid-Holland, the Netherlands, on fallen acorns of *Quercus*, Sept. 1992, *G. Verkley 132*.

*Hymenoscyphus herbarum* (Pers.: Fr.) Dennis. Goudplaat, Wissenkerke, prov. Zeeland, the Netherlands, on dead stems of *Urtica*, Oct. 1992, *G. Verkley 139*.

*Hymenoscyphus imberbis* (Bull.: Fr.) Dennis. Oranjepolder, Voorschoten, prov. Zuid-Holland, the Netherlands, on fallen branches of *Salix*, Oct. 1991, *G. Verkley 97*.

*Hymenoscyphus repandus* (Phill.) Dennis. Groot Berkheide, Wassenaar, prov. Zuid-Holland, the Netherlands, on dead stems of *Epilobium*, May 1990, *G. Verkley s.n.*

*Hymenoscyphus salicellus* (Fr.) Dennis. Hengforder Waarden, Olst, prov. Overijssel, the Netherlands, on dead branches of *Salix*, June 1990, *Piepenbroek 1766* and *1769*; Windesheim, Zwolle, prov. Overijssel, the Netherlands, on dead branches of *Salix*, July 1990, *Piepenbroek 1791*.

*Hymenoscyphus salicinus* (Pers.: Fr.) Kuntze. Harderbos, Zeewolde, prov. Flevoland, the Netherlands, on dead wood, May 1990, *F. Ligtenberg s.n.*; Windesheim, Zwolle, prov. Overijssel, the Netherlands, on fallen branches of *Salix*, Oct. 1990, *Piepenbroek 1827*.

*Pezizella alniella* (Nyl.) Dennis. St. Jansberg, Mook en Middelaar, prov. Limburg, the Netherlands, on fallen female catkins of *Alnus*, March 1992, *G. Verkley 100*.

*Pezizella gemmarum* (Boud.) Dennis. Bos van Bosman, Leiden, prov. Zuid-Holland, the Netherlands, on fallen budscales of *Populus*, April 1992, *G. Verkley 120* and *124*.

*Phaeohelotium subcarneum* (Schum.) Dennis. Fôret de St. Léger, dép. Côte d'Or, France, on wood, Oct. 1990, *J. van Brummelen 7963*.

A detailed clarification of the terminology employed for the wall structure and the stages in ascus development including the corresponding terms for the apical apparatus as used by Bellemère (1977) and Bellemère et al. (1987) has been given elsewhere (Verkley, 1992).

## RESULTS

The extracellular matrix is not noticeably contrasted during the uranyl staining as presently applied. In agreement with earlier work (Verkley, 1992, 1993) the term reactivity is therefore used as an equivalent of electron density in the walls. In the ascoplasm uranyl, osmium and silver deposition determine the electron density. Series of longitudinal median sections of young, immature, mature and dehisced asci were studied. The lateral ascus wall and the apical apparatus are described.

*(text continued on page 319)*

Abbreviations used in figures 1–77. **A**, annulus; **AC**, apical chamber; **AP**, annular protrusion; **AS**, ascospore; **AT**, apical thickening; **av**, apical vesicle; **AW**, ascus wall; **CC**, central cylinder; **E**, epiplasm; **ER**, endoplasmatic reticulum; **G**, glycogen; **IL**, inner layer; **im**, investing membrane; **is**, inner stratum; **L**, lipid body; **M**, gelatinous matrix; **m**, mitochondrion; **mf**, 'myelin figure'; **ms**, middle stratum; **mv**, microvesicles; **N**, nucleus; **OL**, outer layer; **os**, outer stratum; **P**, periascus; **Pa**, paraphysis; **pw**, primary ascospore wall; **SP**, sporoplasm; **SW**, ascospore wall; **sw**, secondary ascospore wall; **ts**, tubular system; **V**, vacuole.

LEGENDS TO FIGURES 1–61:

Figs. 1–6. Longitudinal median sections of ascus apices treated with PA-TCH-SP (bar equals 1 μm). – 1. *Discinella boudieri*. Young elongating ascus. – 2. *Cudoniella acicularis*. Young elongating ascus with rounded apex. – 3–5. *Bisporella pallescens*. 3. Young ascus; 4. mature ascus; 5. dehisced ascus. – 6. *Bisporella sulfurina*. Young ascus.

Figs. 7–12. Longitudinal median sections of ascus apices treated with PA-TCH-SP (bar equals 1 μm). – 7–9. *Bisporella sulfurina*. 7. Immature ascus; 8. mature ascus; 9. dehisced ascus. – 10–12. *Chlorociboria aeruginascens*. 10. Young ascus; 11. immature ascus; 12. mature ascus.

Figs. 13–17. Longitudinal median sections of ascus apices treated with PA-TCH-SP (bar equals 1 μm). – 13–15. *Crocicreas cyathoideum*. 13. Young ascus; 14. immature ascus, advanced stage; 15. dehisced ascus. – 16, 17. *Crocicreas pallidum*. 16. Young ascus; 17. mature ascus.

Figs. 18–24. *Cudoniella acicularis*. Longitudinal median sections of ascus apices treated with PA-TCH-SP (bar equals 1 μm). 18. Young elongating ascus, with conical apex; 19. immature ascus, early stage (primary ascospore wall development); 20. immature ascus, advanced stage (secondary ascospore wall development); 21. mature ascus; 22. idem; 23. dehisced ascus; 24. idem.

Figs. 25–30. Longitudinal median sections of ascus apices and lateral ascus wall (Fig. 30) treated with PA-TCH-SP (bar equals 1 μm). – 25, 26. *Cudoniella clavus* var. *grandis*. 25. Young ascus; 26. immature ascus, advanced stage. – 27–29. *Discinella boudieri*. 27. Immature ascus, early stage (ascospores have just been delimited); 28. immature ascus, advanced stage; 29. dehisced ascus. – 30. *Hymenoscyphus caudatus*. Lateral ascus wall in immature ascus.

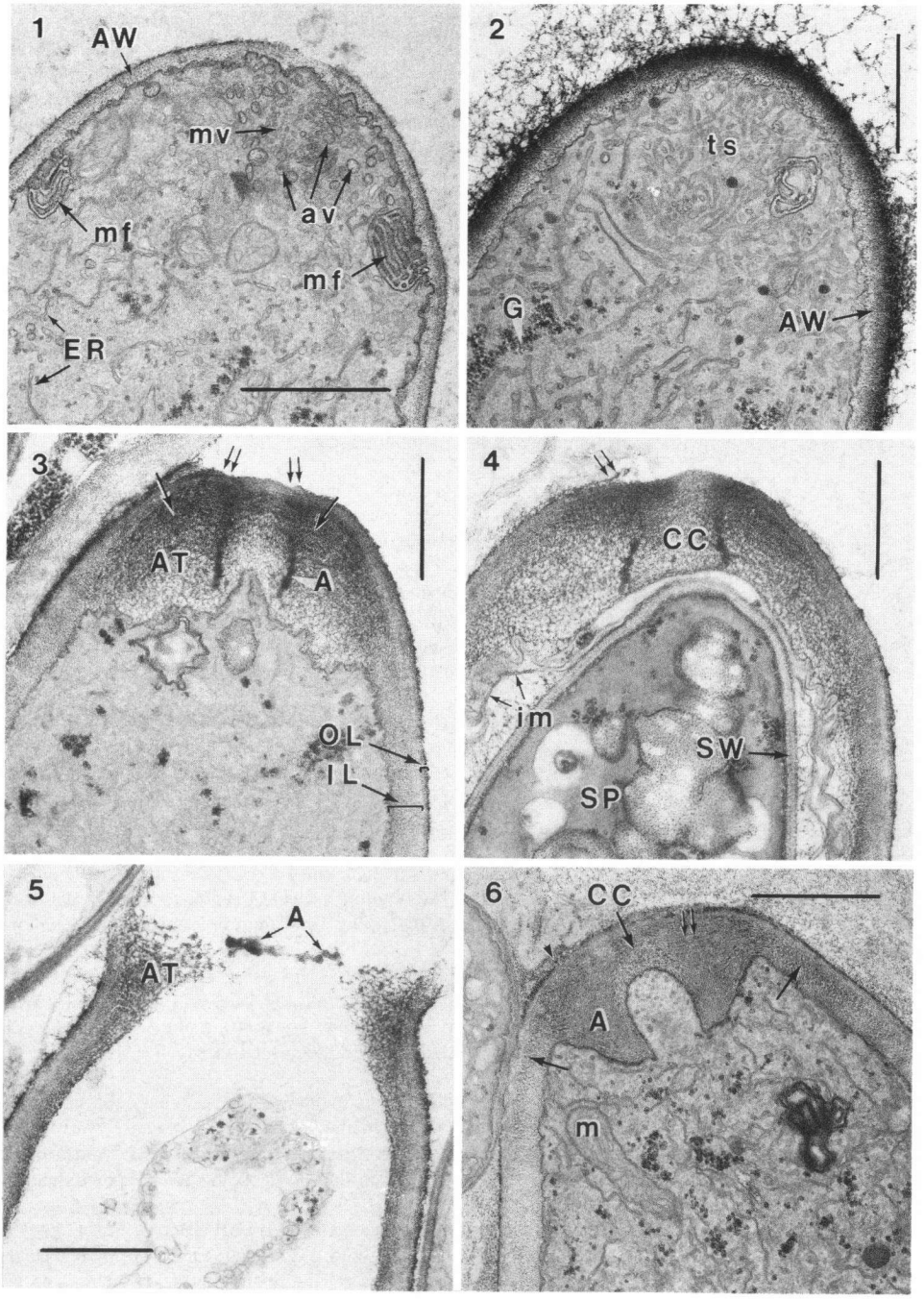
Figs. 31–37. Longitudinal median sections of ascus apices treated with PA-TCH-SP (bar equals 1 μm). – 31–35. *Hymenoscyphus caudatus*. 31. Young ascus; 32. immature ascus; 33. mature ascus; 34. dehisced ascus; 35. idem. – 36, 37. *Hymenoscyphus fructigenus*. 36. Young ascus, during ascospore delimitation; 37. dehisced ascus.

Figs. 38–43. Longitudinal median sections of ascus apices treated with PA-TCH-SP (bar equals 1 μm). – 38–40. *Hymenoscyphus salicellus*. 38. Young ascus; 39. immature ascus; 40. mature ascus. – 41–43. *Hymenoscyphus salicinus*. 41. Young ascus; 42. immature ascus; 43. dehisced ascus.

Figs. 44–49. Longitudinal median sections of ascus apices treated with PA-TCH-SP (bar equals 1 μm). – 44, 45. *Hymenoscyphus consobrinus*. 44. Immature ascus; 45. mature ascus. – 46. *Hymenoscyphus repandus*. Immature ascus. – 47, 48. *Hymenoscyphus imberbis*. Young ascus, during ascospore delimitation; 48. mature ascus. – 49. *Hymenoscyphus herbarum*. Young ascus, shortly before completion of apex formation.

Figs. 50–55. Longitudinal median sections of ascus apices treated with PA-TCH-SP (bar equals 1 μm). – 50–52. *Hymenoscyphus herbarum*. 50. Immature ascus, early stage; 51. immature ascus, advanced stage; 52. dehisced ascus. – 53–55. *Pezizella alniella*. 53. Young ascus; 54. immature ascus; 55. mature ascus.

Figs. 56–61. Longitudinal median sections of ascus apices treated with PA-TCH-SP (bar equals 1 μm). – 56–58. *Pezizella gemmarum*. 56. Young ascus; 57. mature ascus; 58. dehisced ascus. – 59–61. *Phaeohelotium subcarneum*. 59. Immature ascus, early stage; 60. mature ascus; 61. dehisced ascus.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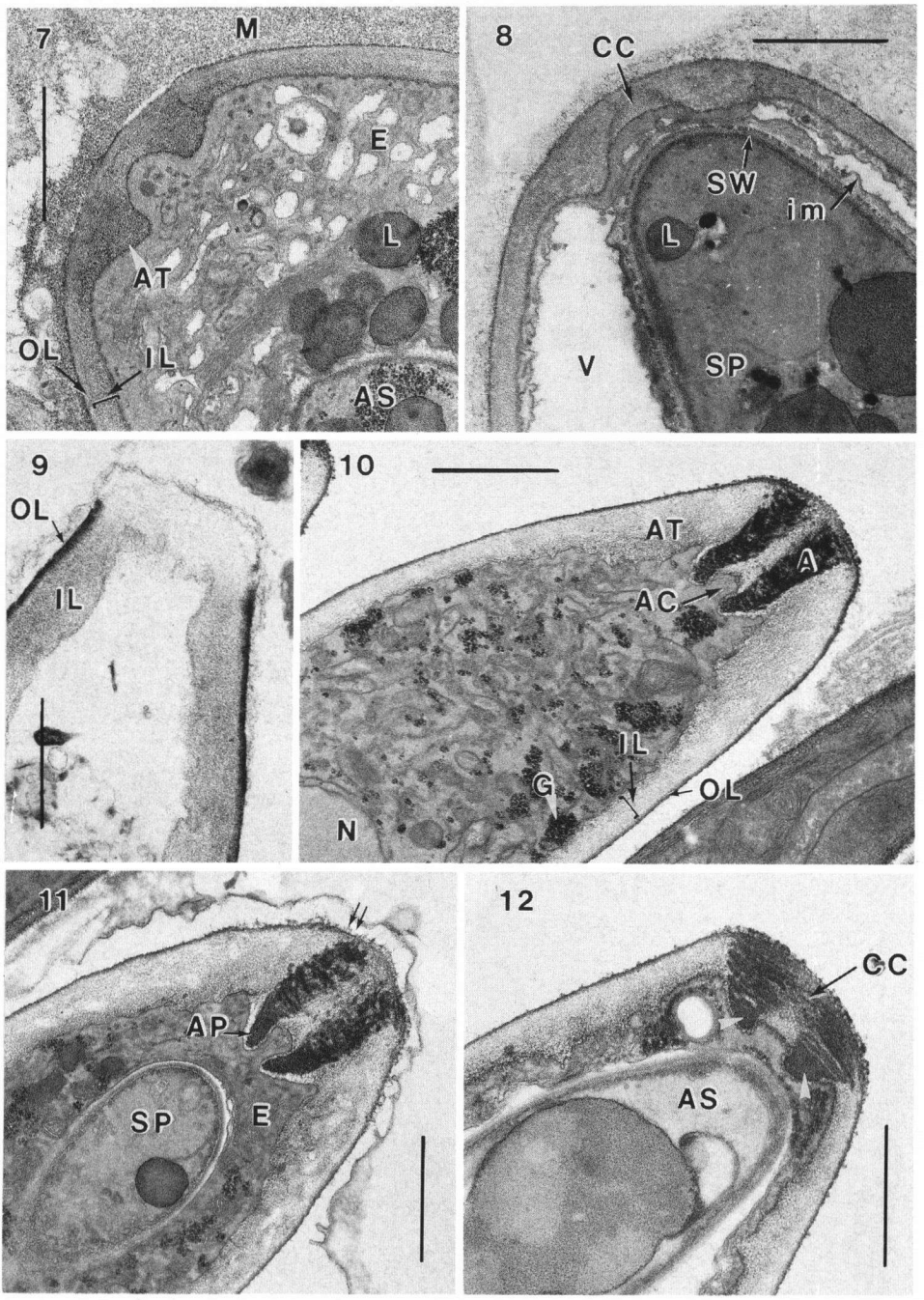
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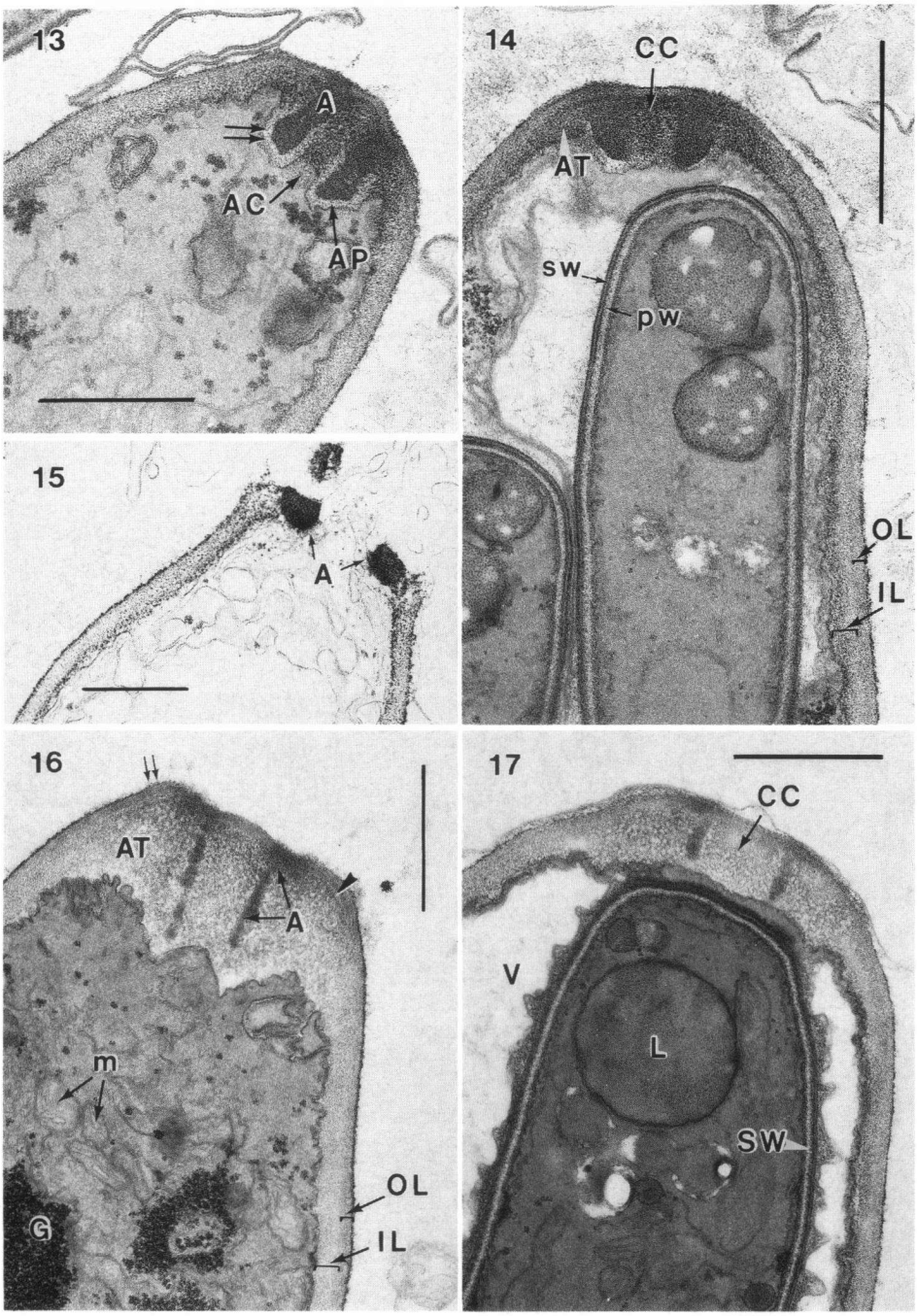
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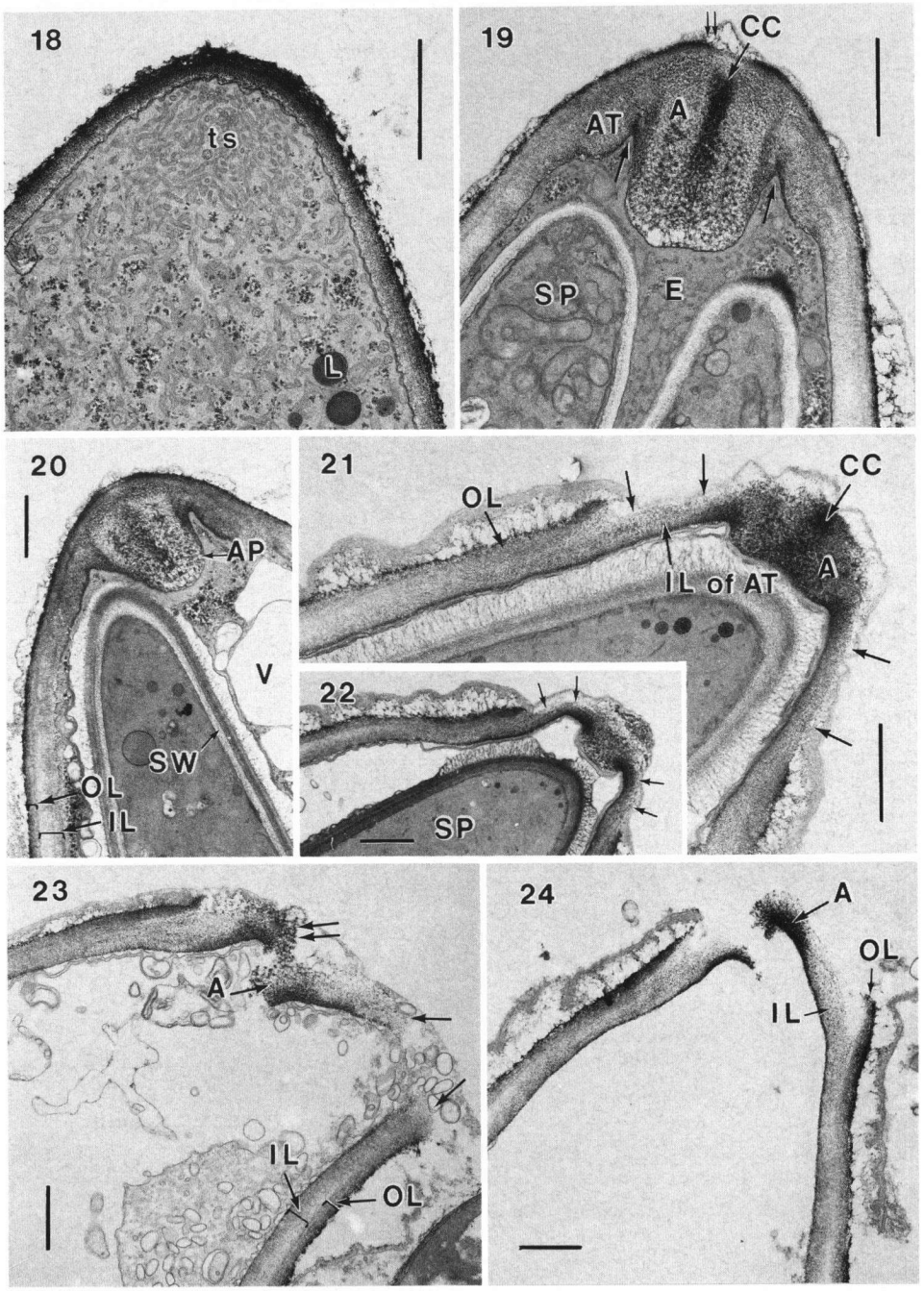
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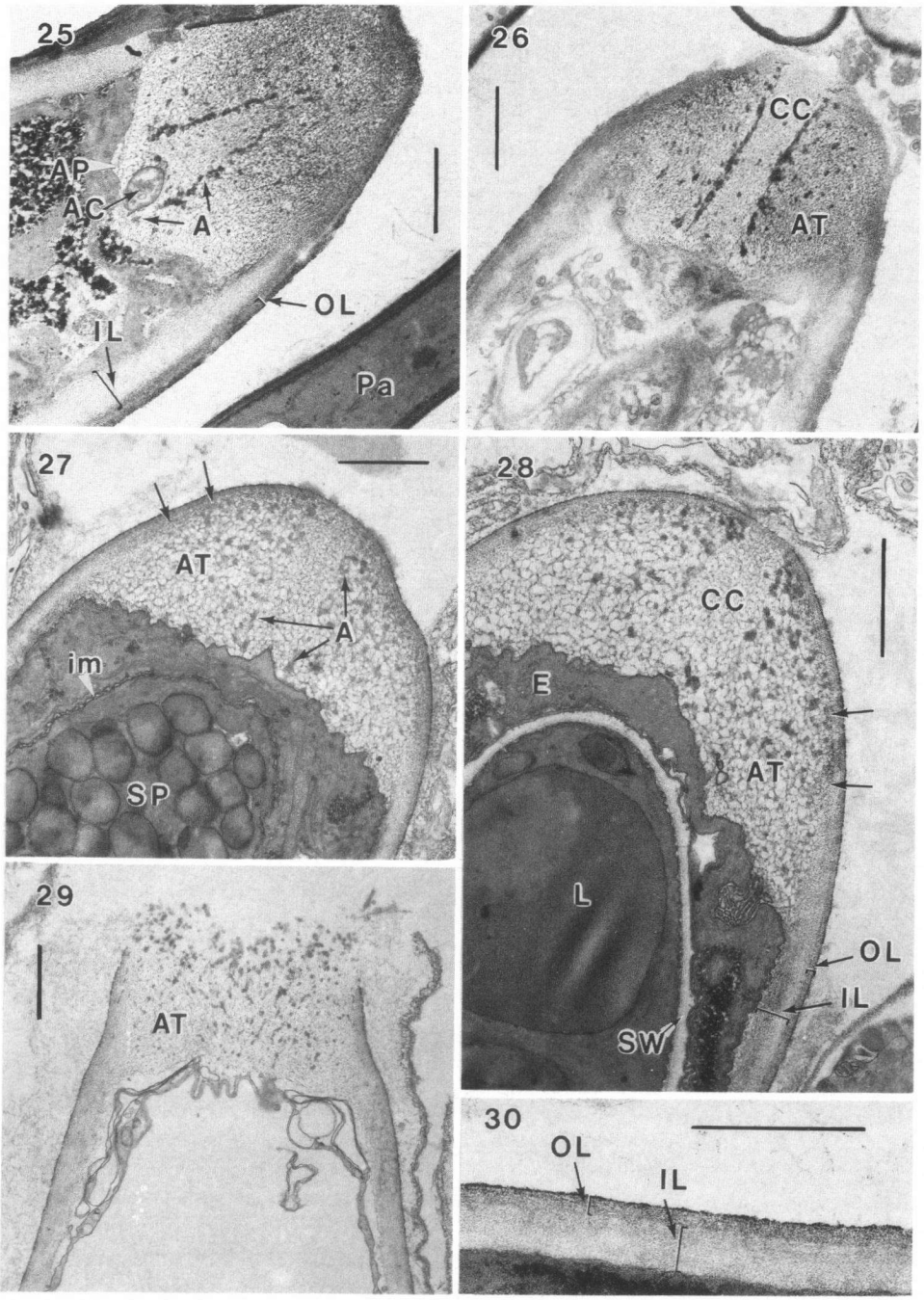
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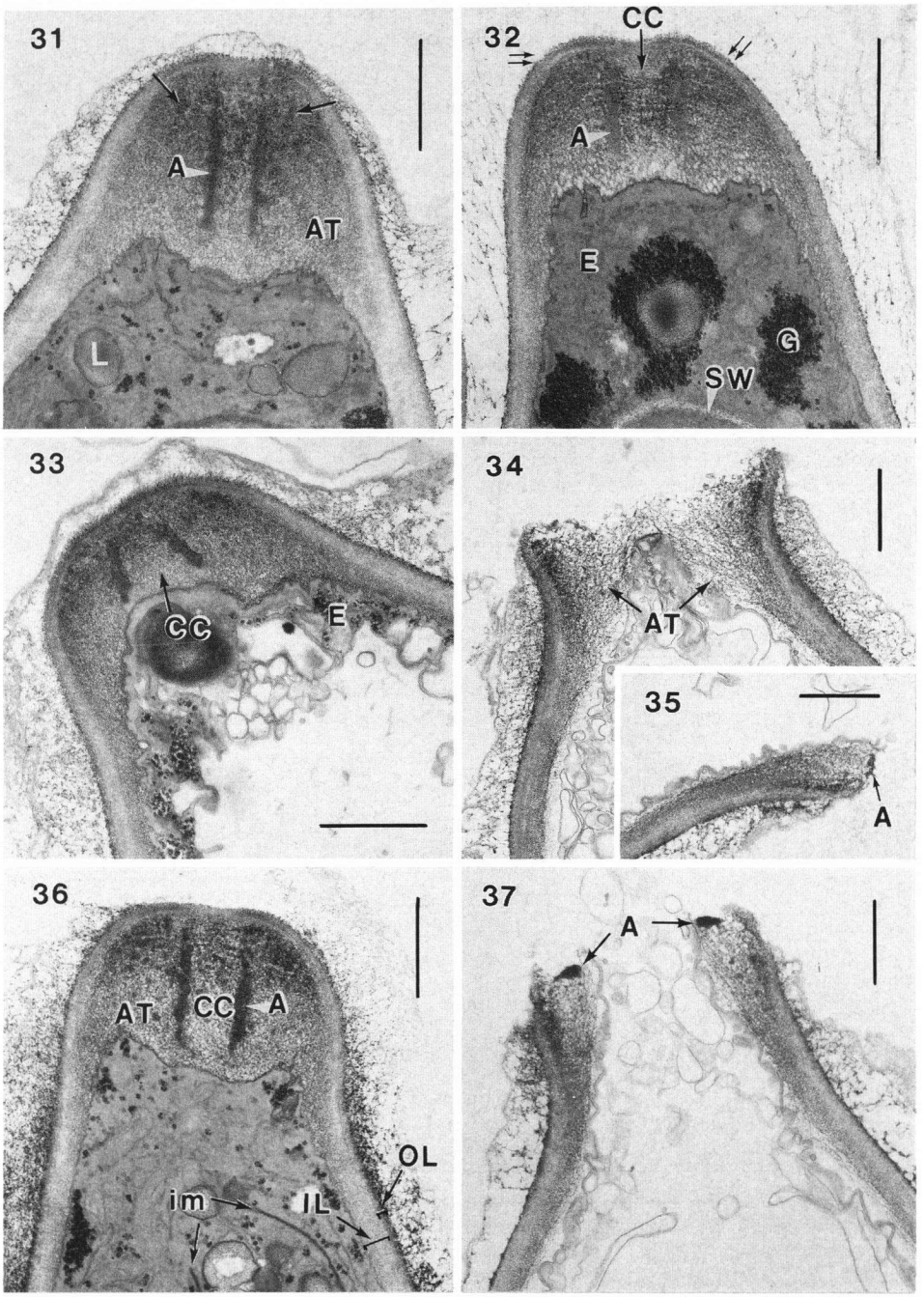
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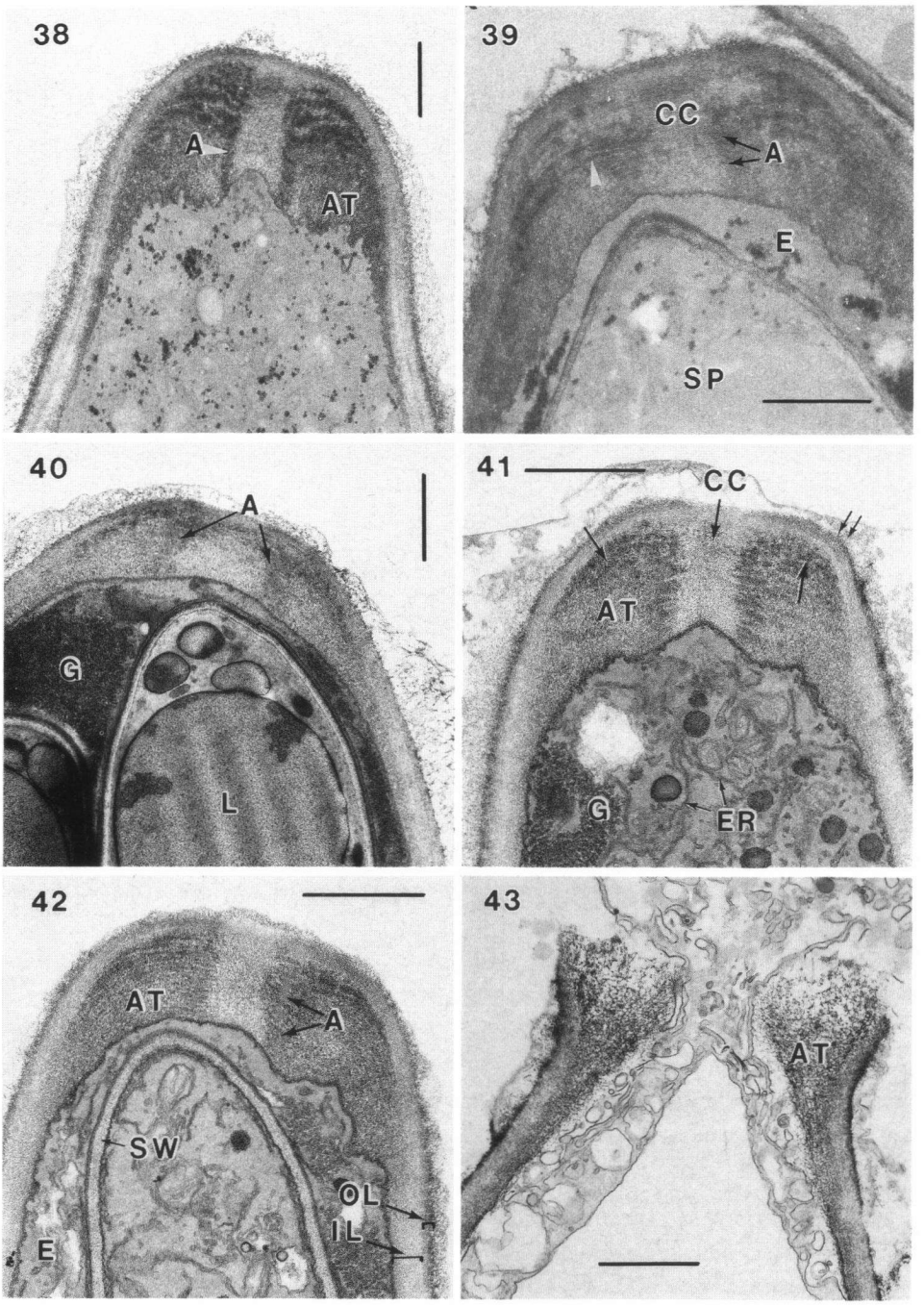
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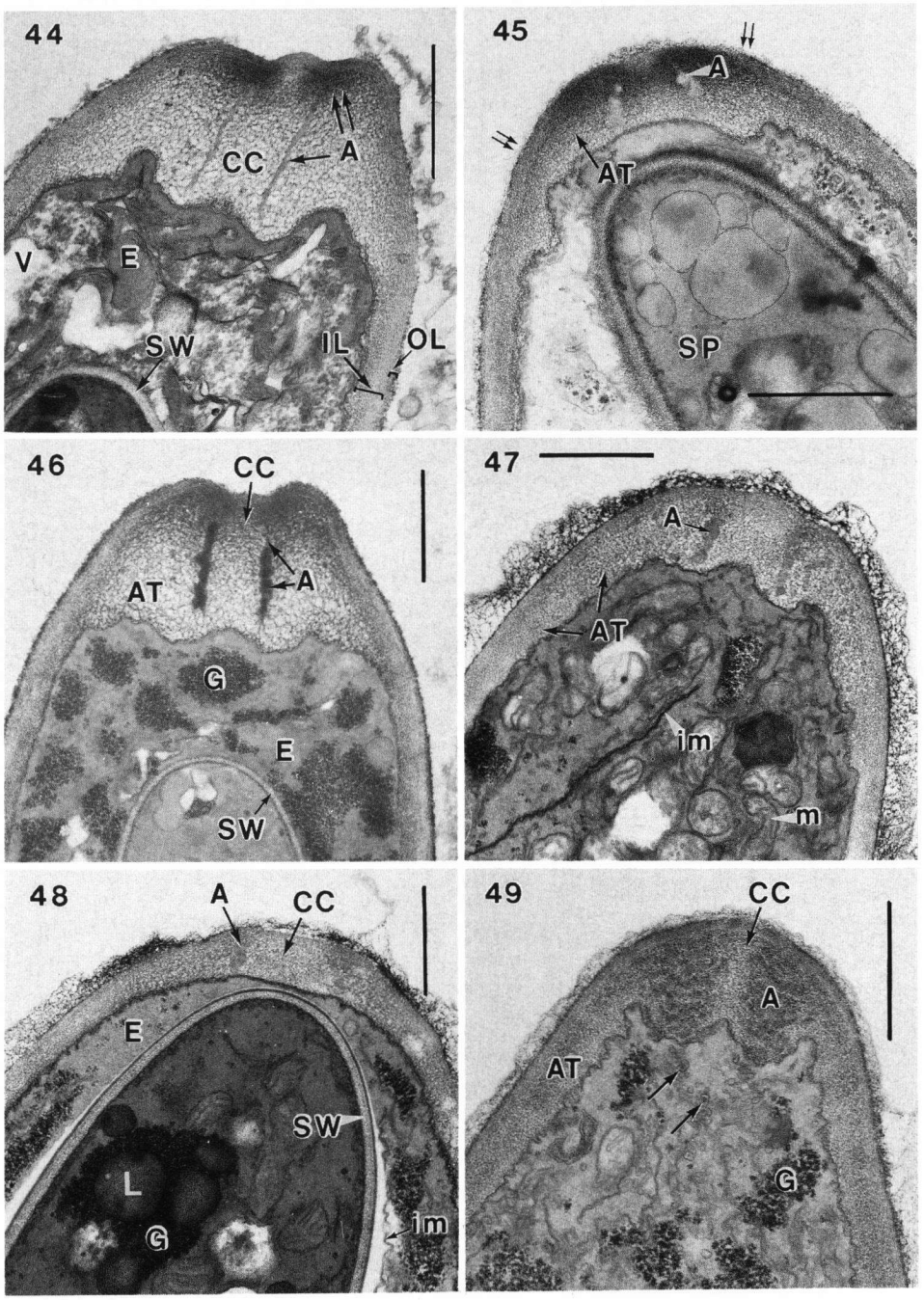
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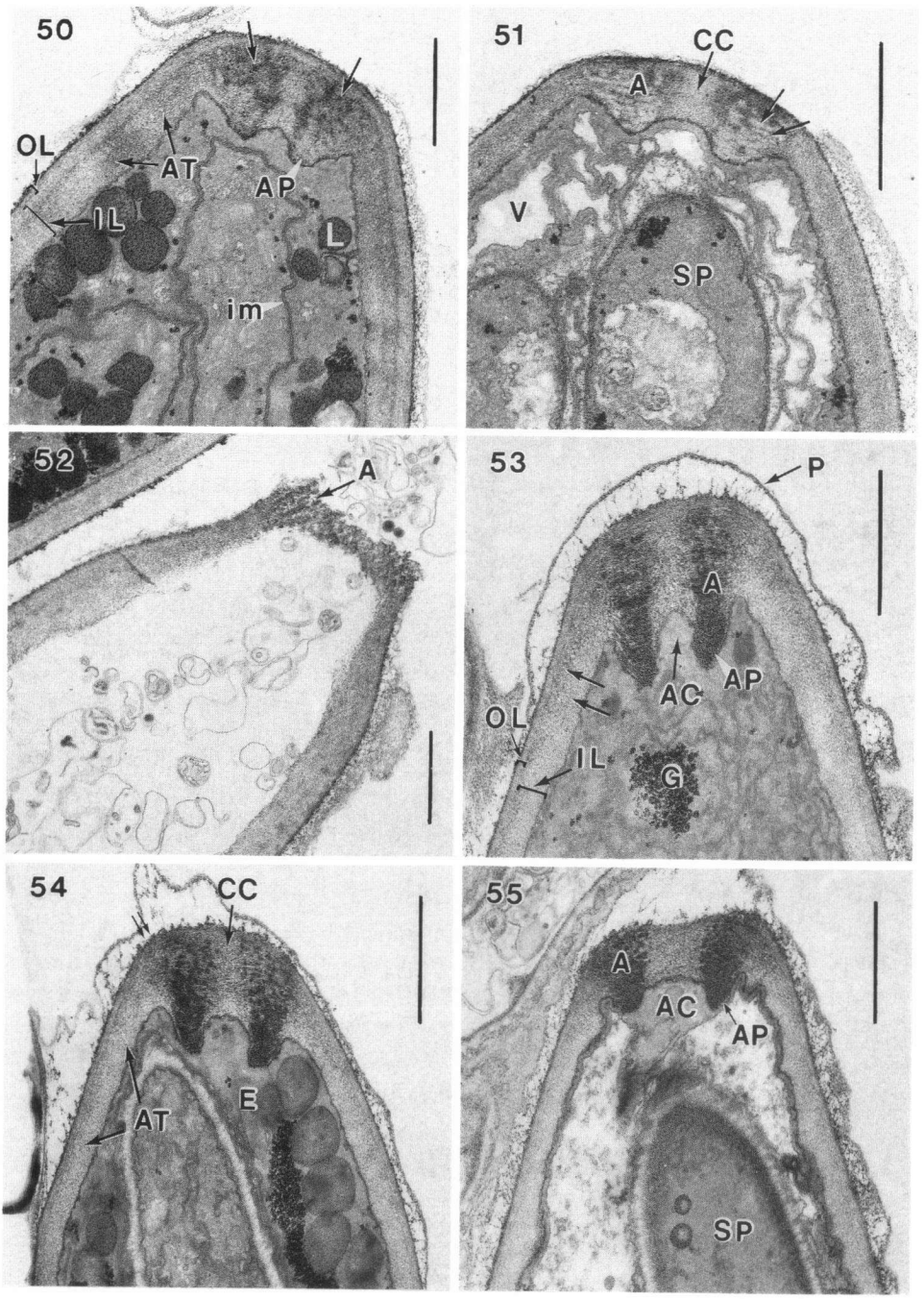
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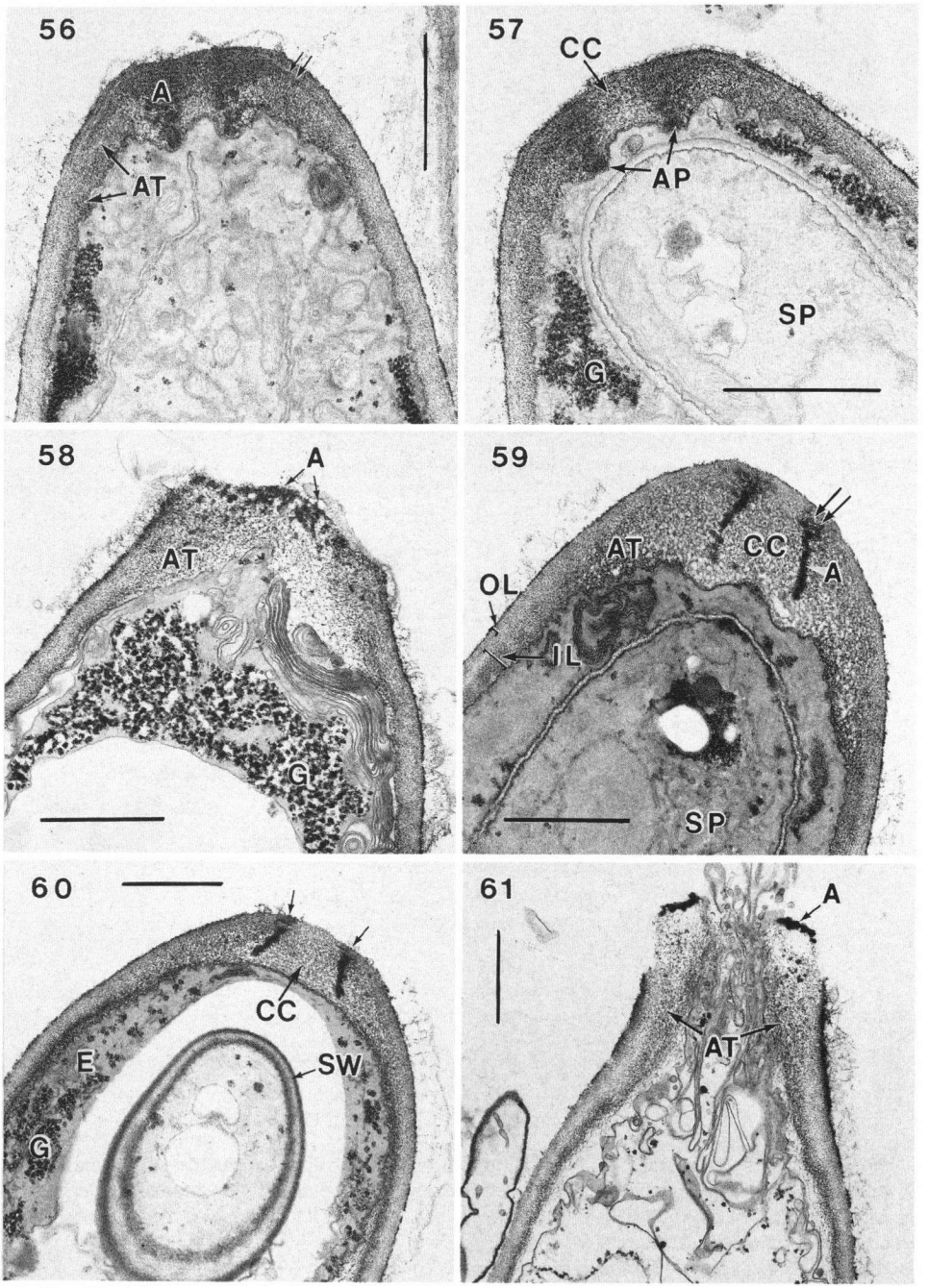
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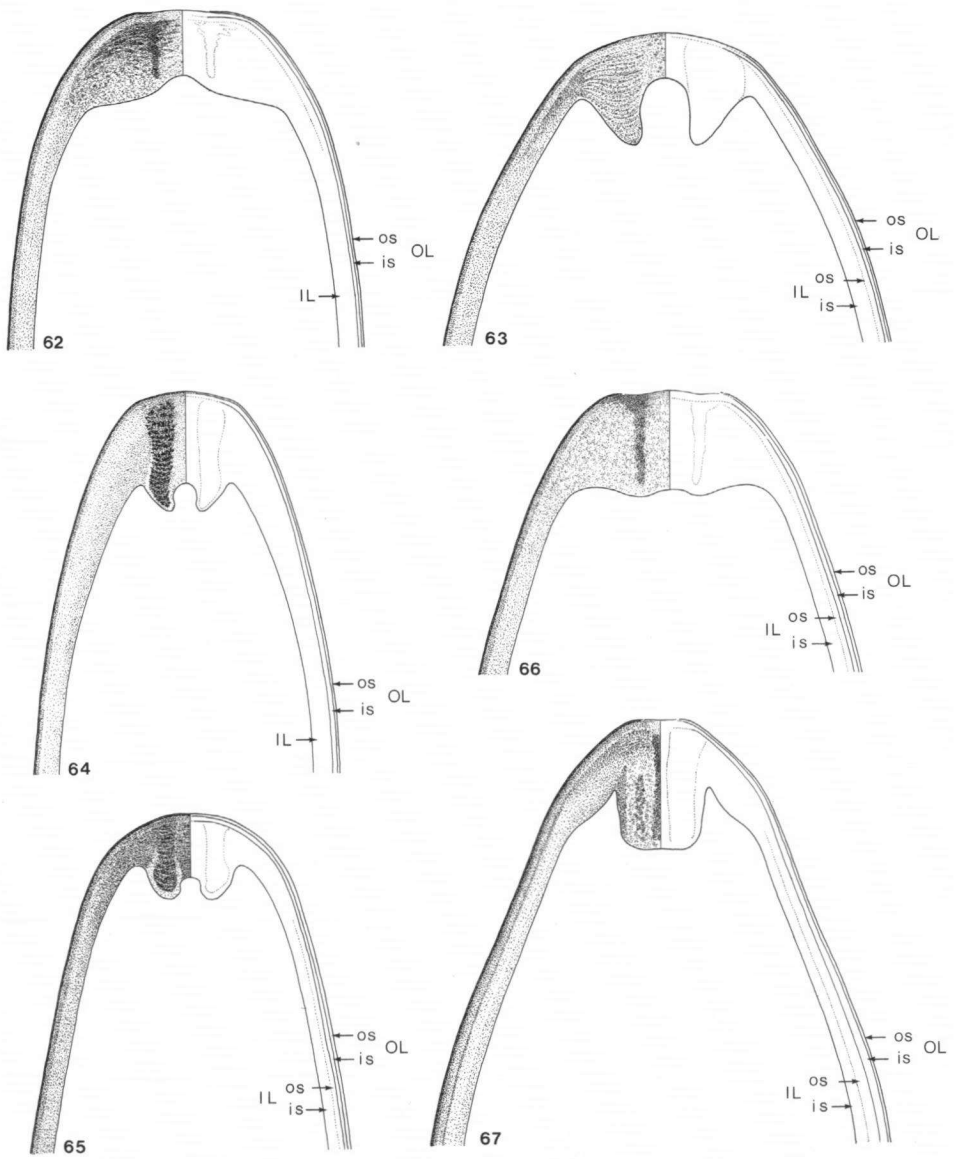
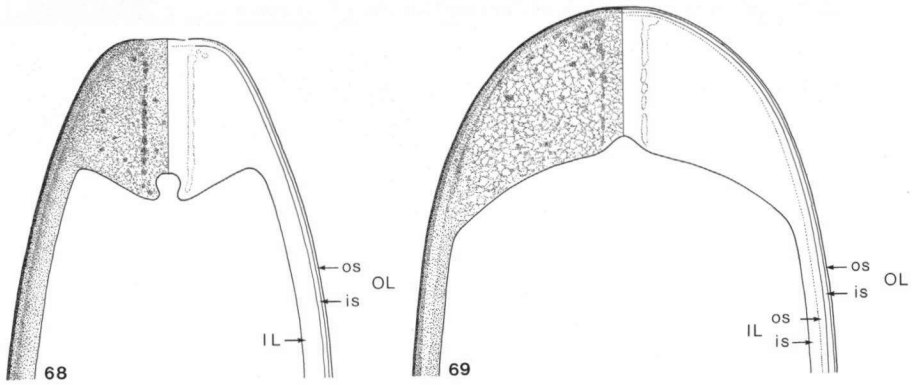



Figs. 62–67. Diagrammatic schemes of median images of the ascus apical apparatus and subapical wall, showing relative PA-TCH-SP reactivity on the left half, and corresponding interpretation of layers, strata and annular region on the right half of each scheme. – 62. *Bisporella pallescens*. Young ascus. – 63. *Bisporella sulfurina*. Young ascus. – 64. *Chlorociboria aeruginascens*. Young ascus. – 65. *Crocicreas cyathoideum*. Young ascus. – 66. *Crocicreas pallidum*. Young ascus. – 67. *Cudoniella acicularis*. Young ascus.




Figs. 68, 69. Diagrammatic schemes of ascus apical apparatus and subapical wall. – 68. *Cudoniella clavus* var. *grandis*. Young ascus. – 69. *Discinella boudieri*. Young ascus.

## GENERAL OBSERVATIONS

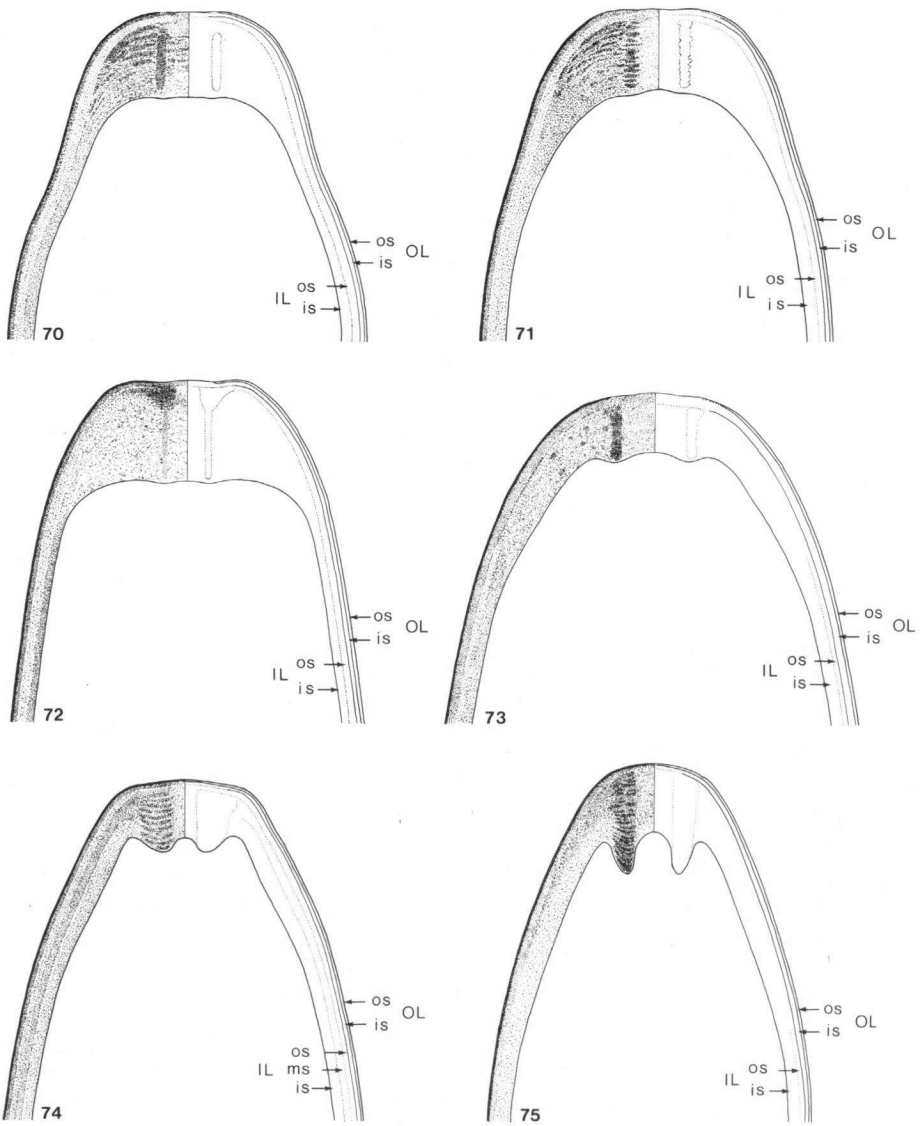
In the species studied it is exceptional to encounter all four stages of ascus development in a single ascoma. For example, in a single apothecium some advanced immature and many mature and dehisced asci of a first 'wave' are encountered together with many young, still elongating asci of a second 'wave'. A certain stage can therefore be missing in some of the species studied.

All species develop an ascus wall with two layers, of which only the inner one increases in thickness at the apex. In dehisced asci the outer layer of the wall remains intact much longer than the inner layer which tends to disintegrate soon after dehiscence.

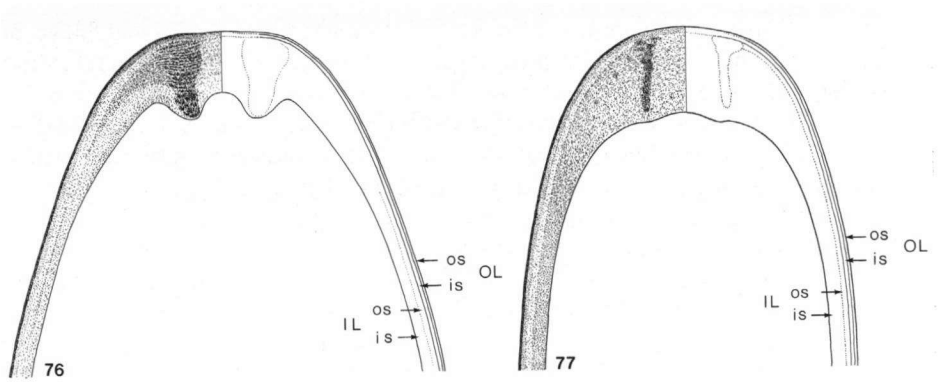
A cap-like layer of reactive material over the apical and subapical wall ('periascus') is found in the majority of asci studied in the species *Cudoniella acicularis*, some species of *Hymenoscyphus* (*H. imberbis*, *H. fructigenus*, *H. salicinus*, *H. salicellus*), *Pezizella alniella* and *Bisporella sulfurina*. In these species this material is clearly associated with the apical wall and is not part of the reactive layer that covers the hymenium as a whole and is observed in other species too. Both types of layers appear to be dislocated quite easily and it is sometimes difficult to discriminate between them or to determine their origin. Deeper within the hymenium between asci and paraphyses a strongly reactive matrix is found in *Pezizella alniella*, *P. gemmarum*, *Discinella boudieri*, *Crocicreas cyathoideum*, *Cudoniella acicularis*, *Chlorociboria aeruginascens*, *Bisporella pallescens*, and *B. sulfurina*.

The young, rapidly elongating ascus initial shows a rounded apex, with in the apical ascoplasm a circular area containing numerous predominantly small vesicles ('microvesicles', Fig. 1), surrounded by an area with predominantly larger vesicles ('apical vesicles', Fig. 1). After the ascus has reached about 80% of its ultimate length at maturity (measured in 1 µm sections of fixed material), apex formation starts and the apical apparatus is formed.

*Cudoniella acicularis* differs in these respects from the other species. Firstly, when the young elongating ascus has reached 50–60 % of its ultimate length, its apex changes shape from rounded to conical. Secondly, the ascoplasm in both rounded and conical




Figs. 70–75. Diagrammatic schemes of ascus apical apparatus and subapical wall. – 70. *Hymenoscyphus caudatus*. Immature ascus. – 71. *Hymenoscyphus salicinus*. Young ascus. – 72. *Hymenoscyphus consobrinus*. Immature ascus. – 73. *Hymenoscyphus imberbis*. Young ascus. – 74. *Hymenoscyphus herbarum*. Young ascus. – 75. *Pezizella alniella*. Young ascus.




Figs. 76, 77. Diagrammatic schemes of ascus apical apparatus and subapical wall. – 76. *Pezizella gemmarum.* Young ascus. – 77. *Phaeohelotium subcarneum.* Immature ascus.

apices is largely filled with an extensive system of branched tubular elements, and shows few if any vesicles (Figs. 2, 18). Apex formation starts when the ascus has reached 80–90 % of its ultimate length.

In all species studied ascospore delimitation starts after the apical apparatus has been fully formed.

## SPECIFIC DESCRIPTIONS

### Bisporella pallescens — Figs. 3–5, 62

The ascus apex is rounded to truncate-rounded. In the lateral ascus wall two layers are observed. The outer layer, 50–80 nm thick, consists of a strongly reactive outer stratum and a less reactive inner stratum. The reactivity of the inner stratum may be stronger over the apical apparatus (Figs. 3, 4). The inner layer, 230–260 nm thick, seems also to consist of two strata since the inner half of this layer frequently shows a stronger reactivity, but this is not always the case.

*Young ascus* – The apical apparatus is formed by an increase in thickness of the inner layer which is at first gradual, and then more abruptly towards the tip. The apical thickening shows an inner zone which seems continuous with the possible inner stratum of the inner layer. In this zone discontinuous layers containing strongly reactive material are oriented parallel to the outer face of the wall (single arrows, Fig. 3). Inwards these layers become less densely arranged and then gradually replaced by a network of reactive microfibrils. In the central cylinder a similar pattern is found with an overall decrease in reactivity towards the ascan length axis. The narrow annulus consists of a homogeneous matrix of strongly reactive material. In the upper part this material seems to change gradually into the strongly reactive layers mentioned above. Some of the asci show wall material below the annulus protruding into the ascoplasm. But this protrusion of material is never closely associated with the annular material nor is it observed in later stages. So, strictly there is

no annular protrusion. The annulus is restricted to the inner zone of the apical thickening. In most asci the outer stratum of the outer layer is largely eroded over the central cylinder (double arrows, Fig. 3).

*Immature and mature ascus* – Upon ripening the apical apparatus appears increasingly compressed (Fig. 4). The inner stratum of the outer layer increases in reactivity and its boundary line with the upper part of the annular region becomes less distinct. The outer stratum is partly eroded over the apical thickening as well (double arrows, Fig. 4).

*Dehisced ascus* – The annulus seems to be everted over about a right angle. The annular material of the lower part is often found disconnected from the rest of the apical thickening (Fig. 5). The material of the central cylinder has disappeared.

**Bisporella sulfurina** — Figs. 6–9, 63

The ascus apex is conical. The lateral ascus wall consists of two layers. The outer layer, 50–60 nm thick, is composed of a strongly reactive outer stratum and a less, but variably reactive inner stratum. The inner layer, 250–280 nm thick, consists of two strata also showing a variable reactivity (Figs. 6, 7). The hymenium is covered by a strongly reactive, gelatinous layer.

*Young ascus* – The apical apparatus is formed by an abrupt increase in thickness of the inner layer. The whole of the apical thickening seems occupied by a broad annulus, so, strictly there is no annular protrusion. Within the inner stratum of the inner layer in the subapical region of the lateral wall a zone of increased reactivity is conspicuous (single arrows, Fig. 6). The annulus is composed of a very fine, layered pattern of reactive microfibrils which are oriented parallel to the inner face of the wall (double arrows, Fig. 6). The central cylinder shows a granular, moderate reactivity. The outer layers outer stratum is usually already partly eroded over the central cylinder and annulus at this stage (arrowhead, Fig. 6).

*Immature ascus* – The apex appears to be flattened, and often a circular depression in the outer face is seen over the annulus (Fig. 7). Towards the end of this stage the apical thickening becomes more compressed and the annular reactivity decreases in most cases, of which in some already markedly.

*Mature ascus* – In most asci the annular reactivity decreases. No further change is observed (Fig. 8).

*Dehisced ascus* – In most asci the apical thickening is severely damaged after dehiscence and few remnants of the annular material are found (Fig. 9).

**Chlorociboria aeruginascens** — Figs. 10–12, 64

The ascus apex is narrowly conical and often flattened at its tip. The lateral ascus wall consists of two layers. The outer layer, 40–55 nm thick, contains a highly reactive outer stratum and a less but variably reactive inner stratum (Fig. 10). In the inner layer, 195–210 nm thick, no conspicuous stratification is observed, but there is a gradual increase in reactivity inwards.

*Young ascus* – The apical apparatus is characterized by a gradual increase in thickness of the inner layer over a relatively extensive area, a well-developed annulus and annular

protrusion enclosing an apical chamber (Fig. 10). Also in the apical thickening the inner layer shows a gradual increase in reactivity inwards. The annulus consists of densely packed, discontinuous layers of strongly reactive material. The upper part of the annulus is broader (Fig. 10). The central cylinder shows a fine granular reactivity. In most asci the outer layer is present over the apex.

*Immature and mature ascus* – In most asci the upper part of the central cylinder is somewhat stronger reactive. The annular material in the protrusion now forms a homogeneous, strongly reactive mass (arrowheads, Fig. 12). The apparatus is considerably compressed and the layered aspect of the material in the upper part of the annulus becomes more distinct (Fig. 12). The outer stratum of the outer layer is eroded over most of the apical apparatus (double arrows, Fig. 11).

*Dehisced ascus* – Not observed.

**Crocicreas cyathoideum** — Figs. 13–15, 65

The ascus apex is rounded to conical-rounded. The lateral ascus wall consists of two layers. In the outer layer, 45–65 nm thick, a strongly reactive outer stratum and a less reactive inner stratum are found. The inner layer, 120–130 nm thick, seems to consist of two strata. The inner stratum is variably reactive and usually only conspicuously stronger reactive than the outer stratum in the advanced immature and mature ascus (Fig. 14).

*Young ascus* – The apparatus is formed by an at first gradual increase in thickness of the inner layer towards the apex, followed by a fierce increase in the annular protrusion. It is characterized by an apical thickening and central cylinder which both mainly consist of a matrix of strongly reactive material densely packed in layers. In the broad annulus this material is the most densely packed (Fig. 13). The lower part of this annulus is outlined by an area of weakly reactive material (double arrows, Fig. 13), while its upper part is often difficult to distinguish from the neighbouring parts of the central cylinder and apical thickening. The well-developed annular protrusion encloses an apical chamber. The outer layer is still present over the apex.

*Immature and mature ascus* – At first no change is observed. But after the formation of the secondary ascospore wall has started (Fig. 14), the apparatus becomes more compressed and the strongly reactive material of the annulus gets a more homogeneous, amorphous appearance. In most asci reactivity increases in the central cylinder and upper part of the apical thickening. At maturity a further compression of the apparatus is observed and the apical chamber seems to disappear almost completely.

*Dehisced ascus* – After dehiscence the annulus still appears as a massive ring, that has now been everted over about a right angle. No remnants of the central cylinder remain attached to the annulus (Fig. 15).

**Crocicreas pallidum** — Figs. 16, 17, 66

The ascus apex is truncate-rounded. In the lateral wall two layers are observed. The outer layer, 55–80 nm thick, consists of a strongly reactive outer stratum and a less reactive inner stratum. The inner layer, 170–195 nm thick, is composed of two strata, of which the inner one is the most reactive (Figs. 16, 17).

*Young ascus* – The apical thickening is formed by a gradual increase in thickness of the inner layer (Fig. 16). The annulus is composed of densely packed reactive material. It is narrow in the inner zone of the apical thickening (i.e. the part that is continuous with the inner layer's inner stratum in the lateral wall). In Fig. 16 the outer boundary of this zone is indicated by the arrowhead. The upper part of the annulus, which partly consists of outer layer material also, is broader. In most of the central cylinder the matrix is similar to that in the apical thickening, except for the part formed by the outer layer's inner stratum. Occasionally material protruding into the ascus cytoplasm was observed (Fig. 16). Since such material never contained reactive annular material and was not observed in later stages of ascus development, it cannot be considered an annular protrusion. The outer layer's outer stratum is usually eroded over the central cylinder and partly over the apical thickening as well (double arrows, Fig. 16).

*Immature and mature ascus* – The apparatus is more compressed on further ripening and there is clearly no annular protrusion (Fig. 17).

*Dehisced ascus* – After dehiscence the annulus is everted over about a right angle. The inner layer of the ascus wall disintegrates rapidly after dehiscence, while the outer layer is more persistent.

**Cudoniella acicularis** — Figs. 2, 18–24, 67

The ascus apex is conical. The lateral ascus wall consists of two layers. The outer layer, 150–170 nm thick, consists of a strongly reactive outer stratum and a much thicker, less reactive inner stratum (Figs. 20, 23). The inner layer, 320–370 nm thick, also consists of two strata, of which the inner one is the more reactive. The thickness of the outer layer decreases towards the ascus top.

*Young and immature ascus* – Before apex formation begins the conical apex contains an extensive tubular system (Fig. 18; see also general observations and Fig. 2). The apical thickening consists of two parts which are separated by a circular constriction in the wall (single arrows, Fig. 19). The subapical (distal) part is characterized by a gradual thickening of the inner layer. The apical (proximal) part is characterized by a fierce thickening of the inner layer, and since it is almost completely filled with annular material it can be considered an annular protrusion. There is no apical chamber, and even a depression at the inner face of the wall in the centre of this part of the apical thickening is normally absent. Thus, the narrow central cylinder and the annular protrusion form a complex. Most of the apical thickening consists of a moderately reactive matrix (Fig. 19).

The uppermost part of the annulus contains fine reactive fibrils oriented more or less in layers parallel to the outer face of wall, while the lower part contains randomly distributed patches of strongly reactive material concentrated in the core. The narrow central cylinder contains strongly reactive material, except for the uppermost part where reactivity is low. The outer layer is usually partly eroded over the central cylinder and annulus in the early immature ascus (double arrows, Fig. 19).

In most late immature asci the complex of the annular protrusion and central cylinder is pushed aside by the uppermost ascospore (Fig. 20).

*Mature ascus* – The uppermost ascospore presses the complex of the annular protrusion and central cylinder out of the outer layer, stretching the inner layer material in the

subapical thickening. The expanded inner layer now shows a thickness about equal to that in the lateral wall (arrows, Figs. 21, 22).

*Dehisced ascus* – Three different dehiscence events are recorded. Firstly, in the majority of the asci observed, the expanded inner layer is partly torn (single arrows, Fig. 23). The complex is pushed aside but remains attached to the rest of the wall. Disintegration of the central cylinder seems to occur soon after dehiscence (double arrows, Fig. 23). Secondly, in some asci the opening is formed somewhere through the complex of annular protrusion and central cylinder (not necessarily through the central cylinder), without a distinct eversion of the annular remnants (Fig. 24). Thirdly, in some asci the complex is completely torn off by a circular tearing of the expanded inner layer.

### Cudoniella clavus var. grandis — Figs. 25, 26, 68

The apex is truncate-rounded. The lateral ascus wall consists of two layers. The inner layer, 100–130 nm thick, consists of a strongly reactive outer stratum and a less reactive inner stratum. The inner layer, 260–330 nm thick, seems to consist of only a single stratum (Fig. 25). An outer zone of inwards rapidly decreasing reactivity is found, but it varies in thickness and its boundary is unclear. Further downwards an inner zone of somewhat stronger reactivity is observed.

*Young ascus* – The apical thickening is formed by an abrupt increase in thickness of the inner layer (Fig. 25). Its matrix mainly consists of a fine network of reactive microfibrils. The matrix of the central cylinder is similar in structure. The narrow, discontinuous annulus consists of patches of strongly reactive material. Similar patches are also found scattered throughout the rest of the apical thickening and central cylinder (Fig. 25). There is a distinct annular protrusion surrounding an apical chamber. The reactivity of the outer layer's inner stratum increases over the central cylinder and annulus. The outer stratum is eroded over the central cylinder.

*Immature and mature ascus* – Usually the outer layer is eroded over most of the apical apparatus. No further change is observed (Fig. 26).

*Dehisced ascus* – After dehiscence the annulus is everted over about a right angle.

### Discinella boudieri — Figs. 27–29, 69

The ascus apex is rounded. The lateral ascus wall consists of two layers. The outer layer, 85–100 nm thick, contains a thin, strongly reactive outer stratum and a less reactive inner stratum (Fig. 28). This inner stratum is much thinner in the apical region. The inner layer, 170–225 nm thick, seems to consist of two strata, of which the inner one is more reactive than the outer one (Fig. 28).

*Young and immature ascus* – The inner layer thickens rather abruptly towards the apex. A thin zone of reactive material is found in the apical region at the boundary of the inner layer's outer and inner stratum (arrows, Figs. 27, 28). In the transitional region from the lateral wall to the apical thickening the matrix is converted from a fine granular reactivity into a regular network of reactive microfibrils (Figs. 27, 28). The apical thickening appears to be swollen, since its inner boundary with the ascoplasm is rather irregular. Patches of concentrated reactivity are found throughout the apical thickening. In the uppermost part

of the apical thickening and near the boundary line with the central cylinder these patches occur more frequently and constitute a diffuse, discontinuous annulus (Fig. 27). A similar but denser network of reactive microfibrils occurs in the central cylinder. Here the patches of concentrated reactivity are only occasionally observed. There is no protrusion associated with the annulus. The outer layer still fully covers the apex in most asci observed. At the advanced immature stage the apical apparatus is more compressed, but no changes are observed in reactivity (Fig. 28). In some asci the outer stratum is eroded over the central cylinder.

*Mature ascus* – Not observed.

*Dehisced ascus* – After dehiscence the apparatus is everted over an angle of about 60–90° (Fig. 29).

**Hymenoscyphus caudatus** — Figs. 30–35, 70

The ascus apex is rounded at the tip and especially characterized by becoming rapidly broader in the subapical region, a feature also observable with the light microscope. In the lateral ascus wall two layers are observed. The outer layer, about 65–80 nm thick, consists of a strongly reactive outer stratum and a less reactive inner stratum (Fig. 30). The inner layer, 190–220 nm thick, consists of at least two strata, of which the inner one is usually the more reactive (Figs. 30, 33).

*Young ascus* – The inner layer forms the apical apparatus as it gradually thickens towards the tip (Fig. 31). The central cylinder and the inner zone of the apical thickening, which is continuous with the inner stratum of the inner layer in the lateral wall, show a granular matrix of moderately reactive material, in which patches of more reactive material are found, especially in the uppermost part (arrows, Fig. 31). The upper part of the central cylinder and the adjacent part of the inner stratum of the outer layer show the same fine granular matrix. The narrow annulus consists of densely packed, strongly reactive material and does not reach into the uppermost part of the apical thickening. There is no annular protrusion (Fig. 31).

*Immature ascus* – In the apical thickening and central cylinder (except the lower part) the patches of strongly reactive material appear to become more orderly arranged in layers oriented more or less parallel to the outer face of the wall (Fig. 32). The annular material remains homogeneously distributed. The outer layer is partly absent over the central cylinder in most asci (double arrows, Fig. 32).

*Mature ascus* – Overall reactivity decreases in most of the apparatus, except for the annulus (Fig. 33).

*Dehisced ascus* – After dehiscence the annulus is everted over about a right angle. It is usually found intact (Fig. 35), but in some asci it seems to have been disrupted during dehiscence (Fig. 34).

**Hymenoscyphus fructigenus** — Figs. 36, 37

The general shape of the ascus apex is the same as observed in *H. caudatus*. In the lateral wall two layers are observed. The outer layer, 70–90 nm thick, consists of a strongly reactive outer stratum covering a less reactive inner stratum. In the inner layer, 200–230

nm thick, two strata are observed in the lateral wall. The inner stratum is usually considerably more reactive than the outer one. In the subapical region however, especially in the young ascus, the reactivity of this inner stratum may vary considerably (Fig. 36).

The apical apparatus agrees with that of *H. caudatus* in structure and reactivity before and after dehiscence (Figs. 36, 37). Here also, the annulus forms a homogeneous mass of strongly reactive material throughout ascus development.

### Hymenoscyphus salicellus — Figs. 38–40

The ascus apex shows a shape similar to that in *H. caudatus*. The stratification of the lateral wall largely resembles that described for *H. caudatus*.

The structure of the apical apparatus also resembles that of *H. caudatus*. However, in the late immature and mature ascus the annulus shows a pattern of thin, strongly reactive layers, which are continuous with the layers found in the rest of the apical thickening (arrowhead, Fig. 39), and in the central cylinder as well, but to a lesser degree than in *H. caudatus* (Figs. 38–40). The mode of dehiscence also agrees with that in *H. caudatus*.

### Hymenoscyphus salicinus — Figs. 41–43, 71

The ascus apex is rounded to truncate-rounded. In the lateral wall two layers are observed. The outer layer, 65–85 nm thick, consists of two strata, a strongly reactive outer stratum and a weaker reactive inner stratum. The inner layer, 190–230 nm thick, seems to consist of two strata which both vary in reactivity (Figs. 41, 42).

*Young ascus* – The inner layer forms the apical apparatus by thickening gradually towards the apex (Fig. 41). The inner zone of the apical thickening consists of a moderately reactive granular matrix. In its upper part patches of strongly reactive material occur which seem to be arranged in layers (single arrows, Fig. 41). The annulus consists of strongly reactive, fine granular material densely arranged in layers. The granular matrix in the central cylinder is less reactive than the one in the inner zone of the apical thickening and does not contain concentrations of strongly reactive material. The inner face of the wall in the central cylinder usually delimits a conical invagination of the wall, but there is no annular protrusion, and therefore no apical chamber. The outer stratum of the outer layer is eroded over most of the apical apparatus at this stage (double arrows, Fig. 41).

*Immature and mature ascus* – The arrangement in layers of the strongly reactive material in the inner zone of the apical thickening becomes more distinct (Fig. 42). At maturity the apical apparatus is considerably compressed.

*Dehisced ascus* – After dehiscence the annulus seems to have been everted over about a right angle, but it is usually difficult to trace the remnants of this structure (Fig. 43).

### Hymenoscyphus consobrinus — Figs. 44, 45, 72

The ascus is rounded to truncate-rounded. The lateral ascus wall consists of two layers. The outer layer, about 50–60 nm thick, consists of a strongly reactive outer stratum and a less reactive inner stratum. The inner layer, about 180–200 nm thick, also consists of two strata, of which the inner one is the more reactive and shows a rough granular appearance (Fig. 44).

*Young ascus* – Not observed.

*Immature ascus* – The inner layer forms the apical apparatus as it gradually increases in thickness towards the apex (Fig. 44). The annulus consists of two structurally different parts. The upper part is relatively broad and contains reactive microfibrils which are densely arranged parallel to the outer face of the wall (double arrows, Fig. 44). The lower part consists of a narrow ring with a homogeneous matrix of fine, granular material. Both parts consist of inner layer material and the lower and most of the upper part of the annulus are embedded in the inner zone of the apical thickening (i.e. the part that is continuous with the inner stratum of the inner layer) (Fig. 44). In the central cylinder and the inner zone of the apical thickening a network of reactive microfibrils occurs which gradually becomes denser from the inner face of the wall outwards. In one of the collections the reactive material was more arranged in layers here. In the uppermost part of the central cylinder there is a small area showing a weaker reactivity. The protrusion of wall material observed below the annulus at this stage is not found in the mature ascus. The outer stratum of the outer layer is eroded over most of the apex.

*Mature ascus* – No marked change is observed. The apical thickening is considerably compressed. The reactivity in the upper part of the annulus and surrounding material increases in some asci (Fig. 45). The outer stratum of the outer layer is further eroded over the apex (double arrows, Fig. 45).

*Dehisced ascus* – After dehiscence the annulus is everted over about a right angle.

### Hymenoscyphus repandus — Fig. 46

The ascus apex is rounded to truncate-rounded. The apical apparatus closely resembles that of *H. consobrinus*, both in structure and reactivity pattern. The lower part of the annulus is generally more reactive, and the reactive material of the outer part of the apical thickening and central cylinder is always arranged in layers (Fig. 46). After dehiscence the annulus is everted over an angle of about 90°.

### Hymenoscyphus imberbis — Figs. 47, 48, 73

The apex is rounded to truncate-rounded. The lateral ascus wall consists of two layers. The outer layer, 55–70 nm thick, is composed of a variably reactive inner stratum covered by a more reactive outer stratum. The inner layer, 180–210 nm thick, is composed of two strata, of which the inner one is usually somewhat more reactive (Fig. 48).

*Young ascus* – The apical apparatus is characterized by a gradual increase in thickness of the inner layer towards the apex over a relatively extended area (Fig. 47). In most asci the wall material protrudes weakly into the ascoplasm in the annular region. The annulus contains a fine and dense granular reactivity, and tends to be somewhat broader and more diffuse in the upper part. Also throughout the rest of the apical thickening patches of relatively stronger reactivity are found. In most asci the outer stratum of the outer layer is absent over the central cylinder and annulus.

*Immature and mature ascus* – During the formation of the secondary ascospore wall the apical apparatus becomes more compressed and the annulus is clearly not associated with any form of protrusion (Fig. 48).

*Dehisced ascus* – After dehiscence the annulus is everted over about a right angle.

**Hymenoscyphus herbarum** — Figs. 49–52, 74

The ascus apex is conical to conical-rounded. The lateral ascus wall consists of two layers. The outer layer, 65–80 nm thick, consists of a strongly reactive outer stratum and a less reactive inner stratum. The inner layer, 235–260 nm thick, consists of three strata that are best visible at the early and mid-immature stages (from spore delimitation untill beginning of secondary spore wall formation, Fig. 50), when each stratum is delimited by a thin line of strongly reactive material. The middle stratum is usually the more reactive. In the lateral wall of the mature ascus these lines are absent and especially the boundary between the outer stratum of the inner layer and the inner stratum of the outer layer is difficult to indicate.

*Young ascus* – Vesicles containing reactive material are found near the plasma membrane at the apex during apex formation (arrows, Fig. 49). The inner layer forms the apical apparatus. The apical thickening shows a poorly developed lower part, while its upper part is well-developed and is almost completely occupied by the annulus. The latter part, which can also be considered an annular protrusion, encloses an apical chamber (Fig. 49). In the annulus layers of strongly reactive material are arranged parallel to the inner face of the wall. The annulus is restricted to the part of the apical thickening that is continuous with the middle and inner stratum of the inner layer in the lateral wall. The central cylinder is moderately reactive. The outer layer is still present over the apparatus at this stage (Fig. 49).

*Immature and mature ascus* – At the early (Fig. 50) and mid-immature stage the fierce layers of strongly reactive material in the annulus are replaced by a pattern of fine microfibrils intermingled with patches of relatively stronger reactivity (arrows, Fig. 50) that are most concentrated in the upper and inner part of the annulus. In the central cylinder the reactivity of the material along the boundary with the annulus increases in some asci. At the advanced immature (Fig. 51) and mature stage this was the case in all asci studied. At this stage most of the annular material looses its reactivity. This material appears in distinct layers within a now strongly reactive matrix (arrows, Fig. 51). The boundary with the outer layer's inner stratum becomes less evident due to a local increase in reactivity in this stratum.

In the mature ascus the outer stratum of the outer layer is eroded over the central cylinder. The lower part of the apical thickening becomes strongly compressed.

*Dehisced ascus* – After dehiscence the annulus is everted over an angle of about 90° (Fig. 52).

**Pezizella alniella** — Figs. 53–55, 75

The apex is conical. The lateral ascus wall consists of two layers. The outer layer, 40–55 nm thick, consists of a strongly reactive outer stratum and a less reactive inner stratum. The inner layer, 155–170 nm thick, consists of two strata, of which the inner one is the more reactive, but the reactivity of both strata tends to vary more in the subapical region (Fig. 53).

*Young ascus* – The inner layer at first gradually increases in thickness over an extended area, and then abruptly forms a distinct annular protrusion which encloses an apical chamber (Fig. 53). In the part of the apical thickening that is continuous with the inner

layer two zones can be observed, a strongly reactive outer zone and a less reactive inner zone (arrows, Fig. 53). The thickness of the outer zone gradually decreases downwards. It is difficult to verify the continuity of these zones with the strata found in the inner layer of the lateral wall. The annulus contains numerous layers of strongly reactive material. In the lower part of this annulus these layers are more closely arranged. The central cylinder shows a granular pattern of reactivity, resembling that observed in most of the apical thickening. The outer layer is present over the apex in most asci.

*Immature ascus* – No marked change is observed. The outer stratum of the outer layer is eroded over most of the apical apparatus (double arrows, Fig. 54).

*Mature ascus* – The apparatus is considerably compressed (Fig. 55). A circular depression in the wall surface is evident over the central cylinder in most asci.

*Dehisced ascus* – Not observed.

**Pezizella gemmarum** — Figs. 56–58, 76

The ascus apex is conical-rounded to rounded and slightly flattened at its tip. In the lateral wall two layers are observed. The outer layer, 45–65 nm thick, consists of a strongly reactive outer stratum and an inner stratum of variable reactivity. However, at the apex there is little difference between both strata. The inner layer, 135–155 nm thick, is homogeneously reactive throughout, but in the subapical part of the wall a line of stronger reactivity seems to reveal two strata (Fig. 56).

*Young, immature and mature ascus* – The inner layer increases in thickness gradually, thus forming a well-developed apical thickening (Figs. 56, 57). The annulus consists of patches of strongly reactive material in the annular protrusion. Upwards it becomes much broader and contains material of equal reactivity which is packed in densely spaced layers (Fig. 57). There the annulus merges with the strongly reactive middle zone of the apical thickening (double arrows, Fig. 56). The innermost part of the apical thickening frequently shows a zone of stronger reactivity. The upper half of the central cylinder is more reactive than the lower half. During the maturation of the ascus no significant change occurs.

*Dehisced ascus* – After dehiscence the annulus is everted over an angle of about 90° (Fig. 58).

**Phaeohelotium subcarneum** — Figs. 59–61, 77

The ascus apex is rounded. The lateral ascus wall consists of two layers. The outer layer, 65–90 nm thick, consists of a strongly reactive outer stratum and a less reactive inner stratum (Fig. 59). The inner layer, 155–170 nm thick, also consists of two strata, of which the inner one is the more reactive (Figs. 59, 60).

*Young ascus* – Not observed.

*Immature ascus* – The inner layer thickens gradually towards the apex over a relatively extended area. The inner zone of the apical thickening and the broad central cylinder consist of a matrix of rough granular material. The outer zone of the apical thickening (continuous with the outer stratum of the inner layer) and the inner stratum of the outer layer show a much finer matrix (Fig. 59). The lower two third part of the annulus is narrow

and consists mainly of a homogeneous, strongly reactive matrix. Approximately the upper one third part of the annulus is broader and consists of scattered patches of similar material (double arrows, Fig. 59). Such patches are found in small numbers in the rest of the apical thickening as well. The protrusion of some wall material just below, but never closely associated with, the annulus is only encountered in asci at this stage and not in mature asci. The outer stratum of the outer layer is eroded over most of the central cylinder and annulus (Fig. 59).

*Mature ascus* – The apical apparatus is considerably compressed (Fig. 60). The outer stratum of the outer layer is further eroded over the apical thickening. The reactivity in the inner stratum of the outer layer has increased over the annular region (arrows, Fig. 60).

*Dehisced ascus* – After dehiscence the annulus is everted over an angle of about 90°, and although it is often disconnected from the other material of the apical thickening, the structure itself is still intact (Fig. 61).

## DISCUSSION

### *The lateral ascus wall*

The two-layered substructure of the lateral ascus wall found in the Hymenoscyphoideae agrees with the one described in several other Leotiales (Corlett & Elliott, 1974; Benny et al., 1978; Verkley, 1992, 1993), Sphaeriales and other pyrenomycetes (Griffiths, 1971; Beckett & Crawford, 1973), and many Pezizales (van Brummelen, 1978). Bellemère (1975, 1977) was the first to study the ascus ultrastructure in the Leotiales in a systematic way using the PA-TCH-SP method of Thiéry (1967). He distinguished four layers in the lateral wall. The outer two, a and b, that were assumed to correspond to the 'exoascus' of Chadefaud (1973), probably correspond to the outer and inner stratum respectively of the outer layer as observed in this study and others (Verkley, 1992, 1993). Whether the layers c and d of Bellemère ('endoascus' of Chadefaud) correspond to the two strata of the inner layer as observed in most species studied presently is more difficult to say. An inner layer with three strata is observed in *Hymenoscyphus herbarum*. Some species of the Sclerotiniaceae also have an inner layer with three strata (Verkley, 1993).

### *The elongating ascus*

The organization of the apical cytoplasm in the elongating ascus initial of all species, except *Cudoniella acicularis*, agrees with that observed earlier in ten species of the Sclerotiniaceae (Verkley, 1993). Therefore, the organization of the apical ascoplasm appears in general to be the same as in the vegetative hyphae of the Ascomycotina (Grove & Bracker, 1970). The occurrence of a tubular system in the apical ascoplasm of *C. acicularis* is taxonomically interesting because characters pertaining to systems of cytoplasmic organelles involved in growth and development would not be expected to vary even within the higher taxa of the fungi. The tubular structures are most likely part of the Golgi system, which normally is less well-preserved in chemically fixed material of fungi (Hoch, 1986).

### *The apical apparatus*

Diagrammatic schemes of the apical apparatus and the subapical wall are depicted in Figs. 62–77. The left half of each scheme shows the relative reactivity in PA-TCH-SP and the right half the interpreted stratification of the wall. The ascus apices of *Hymeno-*

*scyphus fructigenus* and *H. salicellus* resemble the apex of *H. caudatus* (Fig. 70) and the apex of *H. repandus* resembles that in *H. consobrinus* (Fig. 72). The apices of these species are therefore not illustrated. The general shape of the apex as described here in the fixed material generally agrees with the shape of the ascus apex observed in light microscopy. This is not always the case when dried material is rehydrated and observed with the light microscope.

During maturation of the ascus little change is observed in the structure and reactivity pattern of the apical apparatus in most species, especially when compared with those reported in ten species of the Sclerotiniaceae by Verkley (1993). The apex maturation pattern of *Hymenoscyphus herbarum* is characterized by a significant change in the reactivity pattern of the annulus. It may be indicated as an 'inversion pattern' since the annulus reactivity type changes from 'positive' to 'negative'. In *Lanzia luteo-virescens* (Rob.) Dumont & Korf and *Ciboria conformata* (P. Karst.) Svrček of the Sclerotiniaceae this occurs the other way round (Verkley, 1993). In *Bisporella sulfurina* the annular reactivity decreases markedly. In the other species studied presently only small local changes occur in reactivity in addition to a general compression of the apparatus as a whole as maturation progresses. Therefore only one stage is presented in the diagrammatic schemes.

On the basis of the general morphology of the apical apparatus and reactivity pattern of the annulus five main groups can be distinguished. The main characters of these groups and the species referred to them are given below. For group 1 and 2 subgroups of species are outlined as well.

1. Apical thickening without an annular protrusion; reactivity of the annulus in a continuous or discontinuous homogeneous matrix, or in layers; apex rounded to truncate-rounded:

a. Concentration of strongly reactive material in the apical thickening arranged in layers at some stage of apex maturation; annulus continuous and homogeneous: *Bisporella pallescens* (Fig. 62), *Hymenoscyphus caudatus* (Fig. 70), *H. fructigenus*, *H. salicellus*, *H. salicinus* (Fig. 71).

b. Apical thickening extending relatively far downwards, with patches of strongly reactive material; annulus continuous and homogeneous: *Hymenoscyphus imberbis* (Fig. 73), *Phaeohelotium subcarneum* (Fig. 77).

c. Apical thickening without concentrated reactivity; annulus continuous, often with two distinctive parts: *Crocicreas pallidum* (Fig. 66), *Hymenoscyphus consobrinus* (Fig. 72), *H. repandus*.

d. Apical thickening with patches of strongly reactive material; annulus discontinuous: *Discinella boudieri* (Fig. 69).

2. Apical thickening with a well-developed annular protrusion and increasing gradually in thickness over an extended part of the subapical wall; reactivity of the annulus in conspicuous layers; apex conical to conical-rounded:

a. Inner layer in apical thickening with distinct statification at some stage of ascus development; annulus relatively broad, showing 'inversion pattern': *Hymenoscyphus herbarum* (Fig. 74).

b. Inner layer in apical thickening with a strongly reactive zone extending from the upper annular region downwards; annulus relatively broad, no 'inversion pattern': *Pezizella gemmarum* (Fig. 76).

c. Inner layer in apical thickening homogeneously reactive; annulus relatively narrow: *Chlorociboria aeruginascens* (Fig. 64), *Pezizella alniella* (Fig. 75).

d. Apical thickening relatively weakly developed (except for the annular protrusion), and homogeneous strongly reactive as the central cylinder; annulus particularly well-developed in lower part: *Crocicreas cyathoideum* (Fig. 65).

3. Apical thickening fully occupied by an annulus with a reactivity in fine layers (no annular protrusion by definition of Verkley, 1992); apex conical; apex maturation pattern characterized by a marked decrease in annular reactivity: *Bisporella sulfurina* (Fig. 63).

4. Apical thickening increasing in thickness abruptly, with an annular protrusion; annulus narrow and discontinuous; apex truncate to truncate-rounded: *Cudoniella clavus* var. *grandis* (Fig. 68).

5. Apical thickening constricted around a well-developed complex of annular protrusion and central cylinder, central cylinder extremely narrow; apex conical: *Cudoniella acicularis* (Fig. 67).

The taxonomic implications of these results will be discussed more deeply below in 'remarks on taxonomy'.

*The dehiscence mechanism*

The dehiscence mechanism of the species in groups 1, 2 (not observed in *Chlorociboria aeruginascens* and *Pezizella alniella*), and 4 is in agreement with the mechanism found in the family Sclerotiniaceae (Schoknecht, 1975; Bellemère, 1975, 1977; Corlett & Elliott, 1974; Verkley, 1993) and several Leotiaceae (Bellemère 1977; Verkley, 1992). Here, after dehiscence, the annulus is found everted over an angle of about 90°. Usually, the inner side of the annulus is damaged to a lesser or greater degree and no material of the central cylinder is observed associated with the annulus after dehiscence in most species. In the species of Leotiales studied thus far no preformed weakened region in the central cylinder or any (other) indication for active wall decomposition by lysis prior to dehiscence has been found.

Although there is a difference in structure, the central cylinder and annulus form a continuous part of the apical wall. From the structural point of view, terms used in TEM studies like 'pore' (Beckett, 1981), 'pore-plug', or 'plug' (Corlett & Elliott, 1974) are not accurate for Leotiales. The term 'plug', for example, seems to suggest that the central cylinder is segregated from the annulus as a unity during the opening, but this is not the only possible way to be considered. The cylinder may well be internally disrupted during opening, and/or tearing of the annulus may occur to a lesser or greater degree.

What seems to happen next is that the uppermost ascospore everts the annulus on passing through the initially very small opening, stretching the annulus, with the hydrostatic pressure inside the ascus as the driving force. After the tension in the annulus has been relieved, a considerable disintegration of this structure is evident in the ascus after dehiscence. If there were any remnants of the central cylinder attached to the annulus as it is stretched, these are not likely to be found again after dehiscence. After having been everted, the annulus seems to be prevented from returning to its position prior to dehiscence by the swollen wall material of the apical thickening.

The dehiscence mechanism in *Bisporella sulfurina* (group 3) is unknown. The annulus, which in fact is the apical thickening, may be insufficiently preserved during fixation

procedures, or more likely, so severely disrupted during the dehiscence event that it is impossible to determine what happens. Also at the light microscopical level the observations give little information on these very small ascus apices.

In *Cudoniella acicularis* dehiscence occurs in two steps. In the first step the inner layer of the subapical thickening is stretched beyond the erosion rim of the outer layer probably as a result of the increasing internal pressure. The intact outer layer could be expected to prevent this expansion, considering its quite unusual thickness, on average two to three times the thickness of the outer layer in most of the other species. Although such an erosion is common among many other Leotiales and Sclerotiniaceae in particular, an expansion or stretching of the exposed part of inner layer in the apical thickening was never observed in any stage of ascus development in these taxa (Verkley, 1992, 1993). It seems that the expansion in *C. acicularis* is somehow related to a feature of this particular part of the apical thickening, but its ultrastructure is not different from other parts. There are no indications for a physical separation of the outer and inner layer in the sense of a movement of the one along the other. The expansion differs therefore fundamentally from the expansion of the 'endotunica' observed during the dehiscence in bitunicate Ascomycetes (Eriksson, 1981), of which the expansion is also limited in the species of the 'semifissitunicate' type.

In the second step of dehiscence in *C. acicularis* the wall is partly torn normally somewhere in the expansion region next to the complex of annular protrusion and central cylinder. Only rarely this event appears to result in the complete segregation of the complex from the wall. In the rare cases the wall is torn somewhere within this complex no distinct eversion of the annular remnants is observed (occurring in 'semifissitunicate' and 'rostrate' types, Eriksson, 1981). The place in the wall where the opening is started to be formed seems to be determined by the position of the uppermost spore prior to dehiscence. As the internal pressure builds up this spore is pressed against the wall in most cases next to the complex, because there is no apical chamber or depression in the inner face of this complex into which the spore could easily be fixed.

This two step mechanism was also observed in another collection studied alive with the light microscope and can therefore not be an artefact of fixation. In mounts of rehydrated material it is more difficult to observe the undisturbed step-one stage and that is perhaps why this unusual mechanism of dehiscence has not been reported earlier for this rather common species. From the variation in the second step it can be speculated that this species is on the way of developing an apomorphous dehiscence mechanism, new for the order. The similarity in the dehiscence mechanism with some bitunicate ascomycetes is probably based on homoplasy, not on homology.

## Remarks on taxonomy

*Bisporella*

*Bisporella pallescens* and *B. sulfurina* show fundamental differences in the general morphology of their ascus apices. The apex of *B. pallescens* strongly resembles those in species of *Hymenoscyphus* like *H. fructigenus* and *H. caudatus*. The apex in *B. sulfurina* is unlike any of the apices observed in the other species presently studied, but does have

certain characters in common with the ascus apex of *Bulgaria inquinans* (Pers.) Fr. of the Ombrophiloideae sensu Dennis (Verkley, 1992). As in the latter species, the apical thickening in *B. sulfurina* is fully occupied by a broad annulus and a zone of higher reactivity extends downwards into the subapical wall (indicated for *B. inquinans* as 'strate annellogène' by Chadefaud, 1973). But the apex maturation pattern is different and the typical structure of the outer layer of the ascus wall described for *Bulgaria* is not observed in *Bisporella sulfurina* (Verkley, 1992). The ultrastructure of the apical apparatus in *B. citrina* (Batsch.) Korf & S. Carp. seems to differ from those of both *B. pallescens* and *B. sulfurina* (Bellemère et al., 1987). Bellemère et al. state that the apex is similar to the one in *Neobulgaria* Petrak, but this seems insufficiently founded.

The genus *Bisporella*, typified by *B. pallescens*, is predominantly defined by the anatomy of the outer tissue of the receptacle (Korf & Carpenter, 1974). There is, however, a difference in anatomy between *B. pallescens* and *B. sulfurina* which seems to correlate with the differences in ultrastructure of the apices in these species. In *B. pallescens* the hyphae in the well-defined ectal excipulum have thick gelatinized walls which are not clearly delimited from the surrounding gelatinous matrix of reactive polysaccharides (TEM observations) and form a textura oblita. The hyphae in the medullary excipulum are not embedded in such a matrix and form a textura intricata. In *B. sulfurina* the excipular hyphae have thinner walls of which the outer face is more clearly delimited from the surrounding gelatinous matrix. They form a single tissue of textura oblita-intricata throughout the receptacle, except for a zone directly below the hymenium (Baral & Krieglsteiner, 1985; own observations).

*Crocicreas*

Carpenter (1981) monographed *Crocicreas* and defined it in a broad sense. He already noticed the occurrence of two types of ascus apices in the genus, one he called the 'papillate apex' and the other the 'rounded to subtruncate apex'. The first exhibits two thick blue lines in optical cross-section after treatment with Melzer's reagent. It is found in e.g. *C. cyathoideum* and in *C. gramineum*, the type species of *Crocicreas*. The second type shows two thin blue lines at best and, according to Carpenter (1981), sometimes none at all. It is found in e.g. *C. pallidum* and *C. coronatum*, the lectotype species of *Cyathicula* De Not. In *C. pallidum* the lower part of the reactive annulus now observed in TEM corresponds to the thin blue lines observed in light microscopy, while in *C. cyathoideum* rather the whole reactive annulus observed in TEM corresponds to the region blueing in Melzer's. Although he considers characters of hymenial elements, e.g. ascospore size, shape, and septation in general of a more conservative evolutionary nature, Carpenter (1981) does not mention the possible significance of the ascus apical structures. On the characters size and number of teeth on the apothecial margin he comments that these show a considerable intraspecific variation in some species and can in general be considered less conservative. That is why Dennis (1978) and Carpenter (1981) both consider the species with even margins formerly referred to *Phialea* (Fr. ex Pers.) Gill. (Dennis, 1956), but with structurally similar excipulum as the species with dentate margins formerly referred to *Cyathicula*, as congeneric. The occurrence of gel between the characteristic widely spaced hyphae in the ectal excipulum is given the most weight by Carpenter (1981), and he directs the genus to the Ombrophiloideae. He rejects a placement in the Leotioideae or

Hymenoscyphoideae (Korf, 1973), because he does not consider *Leotia* Pers.: Fr. or *Hymenoscyphus* closely related. Yet it appears that it is mainly the occurrence of a gelatinous matrix which presently separates *B. pallescens* and certain species with a 'rounded to subtruncate apex' of *Crocicreas* from *Hymenoscyphus*. Furthermore, there is no evidence in the ultrastructural data to support the hypothesis that *Crocicreas* (including the species with a 'papillate' apex presently investigated, *C. cyathoideum*) is more closely related to the Ombrophiloideae (Verkley, 1992) than to certain genera of the Hymenoscyphoideae. Gelatinization of walls and gel in the extra-mural compartments can occur in various degrees, and even some species of *Hymenoscyphus* have extra-mural fibrillous polysaccharides in the ectal excipulum, but in smaller amounts which can only be demonstrated at the ultrastructural level using PA-TCH-SP (own observations).

From the present data concerning the ultrastructure of the apex it can be concluded 1) that there are probably two distinct groups of species in *Crocicreas*, 2) that both groups show more similarity to other species in the Hymenoscyphoideae than to the species of Ombrophiloideae investigated, and 3) that the group represented by *C. pallidum*, and most probably also by *C. coronatum*, is particularly close to *Hymenoscyphus consobrinus* and some allied species of *Hymenoscyphus*.

*Chlorociboria*

The apical apparatus of *Chlorociboria aeruginascens* agrees well with that of *Chlorociboria aeruginosa* (Pers.: Fr.) Seaver ex Ramamurthi et al. described by Bellemère (1975). Furthermore, it shows interesting similarities in both general morphology and reactivity pattern with the apices in certain species of the Sclerotiniaceae, especially *Poculum petiolorum* (Rob. ex Desm.) Dumont & Korf, but less so with the species of *Ciboria* Fuckel (Verkley, 1993). The apex maturation pattern observed in *P. petiolorum* is not found in *C. aeruginascens*. The species referred to *Chlorociboria* have long been considered closely related to *Ciboria* Fuckel and thus been treated as members of either the Helotiaceae (Rehm, 1896, in 'Ciborieae'; Nannfeldt, 1932, who was uncertain about the position at the subfamily level) or the Sclerotiniaceae later on (Ramamurthi et al., 1957; Korf, 1959; White, 1941). Dixon (1975) concluded in his monograph on *Chlorociboria* that the genus belongs in the family Leotiaceae, tribe Leotieae. If *Chlorociboria* is to be kept in the family Leotiaceae, the genus is most likely one of the closest related to the family Sclerotiniaceae.

*Cudoniella*

The general morphology of the apical apparatus and the dehiscence mechanism in *Cudoniella acicularis* are unlike any of those reported in other Leotiales untill now. In comparison with other Leotiaceae, the morphology of the apical apparatus appears apomorphic, i.e. is more likely to be interpreted as a derived, more specialized form than a primitive form. It is assumed that the central cylinder has been reduced and has lost its function. The apical apparatus in *C. clavus* var. *grandis* is remarkably similar in general morphology to the apparatus in *Ombrophila violacea* Fr. (Verkley, 1992), even more so than *Neobulgaria pura* (Fr.) Petrak. The more diffuse annulus in *C. clavus*, especially in the annular protrusion, may explain why in contrast to *O. violacea* no blueing by Melzer's reagent is observed under the light microscope. The present results demonstrate that the genus *Cudoniella* in its current interpretation is very artificial.

It appears that the light microscopic comparative study of 'inamyloid' ascus apices is of little value from the morphological taxonomic point of view, because it leads to erroneous interpretations.

*Discinella*

In respect of its apical apparatus *Discinella boudieri* seems most closely allied to the species of *Hymenoscyphus* outlined in 'group 1', *B. pallescens* and *Phaeohelotium subcarneum*. *Discinella boudieri* is the type species of a well-established genus which is characterized by an ascus apex blueing weakly in iodine and a preference for terrestrial substrates (Dennis, 1978). The present ultrastructural data support the view of most authors that the genus belongs in the subfamily Hymenoscyphoideae (Korf, 1973) next to *Hymenoscyphus* (Korf, 1973; Dennis, 1956, 1978).

*Hymenoscyphus* and *Phaeohelotium*

*Hymenoscyphus caudatus*, *H. fructigenus* (the type species of *Hymenoscyphus*), and *H. salicellus* agree largely in the ultrastructure of the apical apparatus. They are examples of a group of species in *Hymenoscyphus* that can be characterized by an ectal excipulum of textura porrecta to textura prismatica, fairly specialized ascospores often with tapered distal and 'hooked' proximal ends, with relatively few but large oil-droplets at maturity and with at least in some spores distal and/or proximal 'cilia'. *Hymenoscyphus scutula* (Pers.: Fr.) Phill. is another example of this group, which seems to agree with the one indicated by Dumont (1981) as 'the *H. caudatus* group'. *Hymenoscyphus salicinus*, which shows a similar excipular anatomy but different ascospores, may be closely related considering its apical ultrastructure.

*Hymenoscyphus consobrinus* and *H. repandus* seem to represent another group of species with an excipular anatomy similar to that in the first group, but with simple, always aciliate, ellipsoid to fusoid ascospores (Dennis, 1978; Lizoñ, 1992; own observations). Their apices strongly resemble the apex in *Crocicreas pallidum*.

*Hymenoscyphus imberbis* can be an example of a third group of species in *Hymenoscyphus* developing light-coloured, sessile to short-stalked apothecia with an ectal excipulum of textura globulosa to textura angularis and again simple, aciliate ascospores. This group agrees with 'the *H. epiphyllus* group' as outlined by Dumont (1981). The anatomy of the excipulum in *H. imberbis* is virtually the same as in *Phaeohelotium subcarneum*, and correlates with the resemblance in apical ultrastructure of both species. The only distinct difference concerns the central cylinder, which is broader in *P. subcarneum*, and was noticed by Dennis (1978) as 'broad pore'. Baral & Krieglsteiner (1985) also drew attention to the similarity of these two species in particular. They treated *Phaeohelotium* species in *Hymenoscyphus*, but Lizoñ (1992) proposed to keep *Phaeohelotium* for the brown-spored species (*H. subcarneus* (Sacc.) Kuntze being a different species according to Lizoñ).

*Hymenoscyphus herbarum* differs strongly in its apical ultrastructure from the other species of *Hymenoscyphus* studied as yet, showing resemblance with *Pezizella gemmarum*. The ectal excipulum consists of rectangular cells at a low angle to the surface, the outer hyphae ending in short, smooth-walled, clavate to cylindrical hair-like protuberances. The ascus apex blues intensely in iodine and especially in the upper annular region more than in most other species of *Hymenoscyphus*. Emphasizing the importance of these characters, Baral & Krieglsteiner (1985) reestablished the genus *Calycina* Nees ex S.F.

Gray to accommodate *H. herbarum* [Arendholz (1989) remained doubtful about the identity of Persoon's collections of *Peziza herbarum* Pers. at L.] and several other species they considered congeneric, such as *Pezizella gemmarum* and *P. alniella*. They referred *Calycina* to the Hyaloscyphaceae. Lizoñ (1992) still considers *Peziza herbarum* a species of *Hymenoscyphus*.

From the present data, however, it seems that Baral & Krieglsteiner assembled a still rather diverse group of species doubtfully related to the family Hyaloscyphaceae. There is now sufficient evidence both at LM and TEM level to reject the hypothesis that *H. herbarum* and *H. fructigenus* are congeneric, and it is therefore proposed to accept the recombination of *H. herbarum* to *Calycina* Nees ex S.F. Gray. *Calycina*, considered in a more restricted sense than proposed by Baral & Krieglsteiner (1985), can at present best be placed in the family Leotiaceae, for still very little is known about the ultrastructure of the ascus in Hyaloscyphaceae.

*Pezizella*

The two species of the very large genus *Pezizella* that have been studied ultrastructurally, show a considerable difference in the structure of the apical apparatus. *Helotium gemmarum* Boud. was recombined to *Pezizella gemmarum* by Dennis (1956), although the distinct incrustration on the hair-like protruberances of the ectal excipulum is not characteristic of *Pezizella* in the view of Dennis (1978). Whether *P. gemmarum* is congeneric with *H. herbarum* can best be assessed when the ultrastructure of the apical apparatus of more species will be studied.

The close resemblance of the apices in *P. alniella* and *Chlorociboria aeruginascens* is rather unexpected. It seems reasonable to reject the suggestion of Carpenter (1981, following Müller) that *P. alniella* belongs in *Hymenoscyphus*.

## CONCLUSIONS

In the limited selection of 19 species of the Leotiaceae already a large heterogeneity is recorded in the structure of the ascus apical apparatus, especially when compared with the variation in this structure recorded in ten species of the Sclerotiniaceae (Verkley, 1993).

The characters attributed to the ascus apical apparatus show little variation within groups of species already considered more closely related on the basis of apothecial structure and ascospore characters, such as the type species *Hymenoscyphus fructigenus* and allied species. They may facilitate the arrangement of more natural genera, especially for those ascomycetes with few other distinctive characters.

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